Test Result of Aquatic Toxicity Test

Appendix 6

Marine Eco-Technology Institute Co., Ltd.

Contents of APPENDIX 6

1. SUMMARY REPORT

PROPERTIES AND ACTIONS OF THE PREPARATION

Data on effects on aquatic plant, invertebrate and fish including sensitive and representative organisms

The most sensitive organism for the discharge treated water from the BioViolet™ was the fish , *Paralichthys olivaceus* exposed during 7 days (chronic toxicity test) that gave the LOEC value of 100.00 %, NOEC value of 50.00 %, 7d-LC_{25} value of 80.50 % and 7d-LC_{50} value of higher than 100.00 % for the > 32 PSU discharge treated water . The results are summarized in Tables 1~5.

Growth inhibition test

Algae: Study to assess the effects of the discharge treated water by the BioViolet™ on the diatom, *S. costatum*, for 72 and 96 hours, NOECs, LOECs, 72h-EC_{50} and 96h-EC_{50} value based on the cell densities at the end of the experiment were calculated for > 32 and 3~32 PSU discharge treated water from the BioViolet™. > 32 and 3~32 PSU discharge treated water did not have toxic effect on the *S. costatum.*

Acute aquatic toxicity (Table 2, 3)

Invertebrate: Study to assess the effects of the discharge treated water from the BioViolet™ on the Rotifer, *Brachionus plicatilis,* for 24 hours, NOEC, LOEC and 24h-LC_{50} values based on survival data at the end of the experiment were calculated for > 32 and 3~32 PSU discharge treated water from the BioViolet™. > 32 and 3~32 PSU discharge treated water did not have acute toxic effect on the Rotifer, *B. plicatilis.*

Fish: Study to assess the effects of the discharge treated water from the BioViolet™ on the olive flounder, *Paralichthys olivaceus*, for 96 hours, NOEC, LOEC and 96-LC_{50} values based on survival data at the end of the experiment were calculated for > 32 and 3~32 PSU discharge treated water from the BioViolet™. > 32 and 3~32 PSU discharge treated water did not have acute toxic effect on the olive flounder, *P. olivaceus*.

Chronic aquatic toxicity (Table 4, 5)

Invertebrate: Study to assess the effects of the discharge treated water from the BioViolet™ on the Rotifer, *B. plicatilis,* for 96 hours, NOEC, LOEC and 96h-EC_{50} value based on population growth rate data at the end of the experiment were calculated for > 32 and 3~32 PSU discharge treated water from BioViolet™. > 32 and 3~32 PSU discharge treated water did not have chronic toxic effect on the Rotifer, *B. plicatilis.*

Fish: Study to assess the effects of the discharge treated water from BioViolet™ on the olive flounder, *P. olivaceus*, for 7 days, NOEC, LOEC, 7d-LC_{25} and 7d-LC_{50} value based on survival data at the end of the experiment were calculated for both > 32 and 3~32 PSU discharge treated water from the BioViolet™. > 32 PSU discharge treated water, NOEC, LOEC, 7d-LC_{25} and 7d-LC_{50} value of flounder, *P. olivaceus* chronic toxicity test were 50.00 %, 100.00 %, 80.50% and >100.00%, respectively. For 3~32 PSU discharge treated water, NOEC, LOEC, 7d-LC_{25} and 7d-LC_{50} value were equal to higher than 100.00 %.

Table 1: Growth inhibition test results on > 32 and 3-32 psu de-ballast water from BioViolet™

	Species	Test water (psu)	NOEC	LOEC	LC_{50} and/or EC_{50}		Reference
			Conc. (%)		End point	Conc. (%)	
Algae	*Skeletonema costatum*	> 32	100.00	>100.00	72h-EC_{50}	>100.00	ISO 10253
			100.00	>100.00	96h-EC_{50}	>100.00	
		3-32	100.00	>100.00	72h-EC_{50}	>100.00	
			100.00	>100.00	96h-EC_{50}	>100.00	

Table 2: Acute aquatic toxicity of > 32 psu de-ballast water from BioViolet™

	Species	NOEC	LOEC	LC_{50} and/or EC_{50}		Reference
		Conc. (%)		End point	Conc. (%)	
Invertebrate	*Brachionus plicatilis*	100.00	> 100.00	24h-LC_{50}	> 100.00	ASTM E1440-91
Fish	*Paralichthys olivaceus*	100.00	> 100.00	96h-LC_{50}	> 100.00	OECD 203

Table 3: Acute aquatic toxicity of 3-32 psu de-ballast water from BioViolet™

	Species	NOEC	LOEC	LC_{50} and/or EC_{50}		Reference
		Conc. (%)		End point	Conc. (%)	
Invertebrate	*Brachionus plicatilis*	100.00	> 100.00	24h-LC_{50}	> 100.00	ASTM E1440-91
Fish	*Paralichthys olivaceus*	100.00	> 100.00	96h-LC_{50}	> 100.00	OECD 203

Table 4: Chronic aquatic toxicity of > 32 psu de-ballast water from BioViolet™

	Species	NOEC	LOEC	$LC_{25, 50}$ and/or EC_{50}		Reference
		Conc. (%)		End point	Conc. (%)	
Invertebrate	*Brachionus plicatilis*	100.00	> 100.00	96h-EC_{50}	> 100.00	- ASTM E1440-91 - Janssen *et al.*,1994
Fish	*Paralichthys olivaceus*	50.00	100.00	7d-LC_{25} 7d-LC_{50}	80.50 > 100.00	OECD 212

Table 5: Chronic aquatic toxicity of 3-32 psu de-ballast water from BioViolet™

	Species	NOEC	LOEC	$LC_{25,50}$ and/or EC_{50}		Reference
		Conc. (%)		End point	Conc. (%)	
Invertebrate	*Brachionus plicatilis*	100.00	> 100.00	96h-EC_{50}	> 100.00	- ASTM E1440-91 - Janssen *et al.*,1994
Fish	*Paralichthys olivaceus*	100.00	> 100.00	7d-LC_{25} 7d-LC_{50}	> 100.00 > 100.00	OECD 212

2. FINAL REPORT

2.1 Algae Growth Inhibition Test

2.1.1 ANNEX 1

***Skeletonema costatum*, Growth Inhibition Test - 72 hours**

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FINAL REPORT

Aquatic Toxicity For > 32 & 3-32 psu discharge treated water of BioViolet™

- *Skeletonema costatum*, Growth Inhibition Test – 72 hours

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GLP STATEMENT AND SIGN

Study title: Algae growth inhibition test-72h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward *Skeletonema costatum*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

All procedure of this study was carried out following GLP regulation:

- "OECD Principles of Good Laboratory Practice"
  Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

This study was conducted on the basis of 'the Study Plan' complied and the procedure described in 'the Study Plan' for this report was carried out under 'the Responsibility of Study Director'. This report was built on the basis of raw data from the study.

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.

Study Director Myung-Baek Shon (signature)

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.

Project Officer Min Ho Son (signature)

GUARANTEE OF ASSURANCE

Study title: Algae growth inhibition test-72h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward *Skeletonema costatum*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

Quality assurance personnel of Marine Eco-technology Institute Co., Ltd., inspected as follows. Inspection was carried out on the basis of the Standard Operation Procedure and each inspection results were reported to Study Director and Test Facility Manager.

Types of inspection and the date inspection results were reported to the Test Facility Management and the Study Director as follow:

Type of Inspections	Date of Inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011. 02. 28	2011. 02. 28
Test organisms	2011. 10. 08 / 10. 29	2011. 10. 10 / 10. 31
Storage of Test substance	2011. 10. 10 / 10. 31	2011. 10. 10 / 10. 31
Preparation of Test substance	2011. 10. 11 / 11. 01	2011. 10. 11 / 11. 01
Exposure	2011. 10. 11 / 11. 01	2011. 10. 11 / 11. 01
Observation	2011. 10. 12 / 11. 02	2011. 10. 12 / 11. 02
Record of raw data	2011. 10. 12 / 11. 02	2011. 10. 12 / 11. 02
Draft of final report	2011. 12. 23	2011. 12. 23
Final report	2011. 12. 30	2011. 12. 30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Jin Hee Kim (signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

1. SUMMARY

The purpose of this study was to assess the toxic effects of the > 32 and 3-32 psu discharge treated water from the BioVioletTM on the growth of diatom, *Skeletonema costatum* during the 72 hours.

The test substances were divided into two different salinities, the one was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. Diatom, *Skeletonema costatum* inoculated 3,380±387 cells/mL (mean±S.D) at > 32 psu and 2,903±387 cells/mL (mean±S.D) at 3-32 psu on the exponential phase was incubated at the various test concentrations (0.00 % (non-discharge treated water), 6.25, 12.50, 25.00, 50.00 and 100.00 % (discharge treated water)) for 72 hours. The cell density was counted daily by measuring chlorophyll *a* with 90% acetone using Tuner Designs AU-10 including the control. To obtain the regression equation between chlorophyll *a* and cell density, cell count in the control was done by manual cell counting used the microscope with counting chamber. The raw data of growth from this experiment was used for calculation of inhibition of growth rate and values of 72h-EC50, LOEC and NOEC.

For > 32 psu and 3-32 psu discharge treated water, growth curves based on the cell density calculated from chlorophyll *a* value show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (72 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -7.53 to 4.55 percent in >32 psu and from -32.66 to 0.07 percent in 3-32 psu discharge treated water.

For >32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 72h-EC50 calculated from growth rate were equal to or higher than 100.00 % discharge treated water at the end of the experiment.

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have toxic effects on the growth of diatom, *Skeletonema costatum*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Reference toxicant test	2011. 10. 04 ~ 10. 07
Acclimation	2011. 02. 28 ~ 10. 29
Pre-culture for test	2011. 10. 29
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01
Evaluation of cell density	2011. 11. 01 ~ 11. 04
Evaluation of chlorophyll *a*	2011. 11. 02 ~ 11. 05
Experimental completion	2011. 11. 04
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 10. 08
Pre-culture for test	2011. 10. 08
Collect of sample	2011. 10. 10
Experimental start	2011. 10. 11
Exposure date	2011. 10. 11
Evaluation of cell density	2011. 10. 11 ~ 10. 14
Evaluation of chlorophyll *a*	2011. 10. 12 ~ 10. 15
Experimental completion	2011. 10. 14
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For satisfactory correlation with biomass, chlorophyll *a* and cell density in the control were analyzed by linear correlation analysis with Excel program and the greatest R^2 value was yielded. Cell density was calculated by equation from the linear correlation analysis.

For > 32 psu and 3-32 psu discharge treated water, Shapiro-Wilk's Test and Bartlett's Test were respectively used to normality and homogeneity of variance of specific growth rate. Dunnett's Test was used to determine the NOEC and LOEC. Differences in all tests were considered to be significant at α=0.05. TOXCALC 5.0 program (Tidepool scientific software, USA) was used for above statistic analysis.

4. RESULTS

4.1 Reference toxicant test (Table 1)

For reference toxicant test with diatom, *S. costatum*, the value of 72h-EC50 was 3.4 mg/L as required by the precision object that ranged from 1.4 mg/L to 3.6 mg/L.

4.2 pH values (Table 2, 3)

For >32 psu discharge treated water, the initial pH value was ranged from 7.74 to 7.78 and the final one was ranged from 8.99 to 9.11. For 3-32 psu discharge treated water, the initial pH value was ranged from 7.65 to 7.73 and the final one was ranged from 9.02 to 9.20.

4.3 Biomass

4.3.1 Discharge treated water - > 32 psu (Table 4, 6, Figure 1)

The values of the chlorophyll *a* and cell density had functional relationship indicated by the equation Y=1.25X+2.59 (R^2=0.98, n=16, P<0.001). Therefore, cell density in the each test concentration including control was calculated from the equation. The initial cell density in the test concentrations was ranged from 2,996 to 4,414 cells/mL (mean±S.D: 3,380±387 cells/mL). The mean cell density in the control at the end of the experiment was 396,680 cells/mL. The mean cell densities in the each test concentration at the end of the experiment, 6.25, 12.50, 25.00, 50.00 and 100.00 % were ranged from 367,447 to 458,904 cells/mL.

4.3.2 Discharge treated water - 3-32 psu (Table 5, 7, Figure 2)

The values of the chlorophyll *a* and cell density had functional relationship indicated by the equation Y=1.21X+2.91 (R^2=0.99, n=16, P<0.001). Therefore, cell density in the each test concentration including control was calculated from the equation. The initial cell density in the test concentrations was ranged from 2,404 to 3,894 cells/mL (mean±S.D: 2,903±387 cells/mL). The mean cell density in the control at the end of the experiment was 618,762 cells/mL. The mean cell densities in the each test concentration at the end of the experiment, 6.25, 12.50, 25.00, 50.00 and 100.00 % were ranged from 684,726 to 751,111 cells/mL.

4.4 Growth curve

4.4.1 Discharge treated water - > 32 psu (Fig. 3)

For the > 32 psu discharge treated water, growth curves show that the growth at all test concentrations of the discharge treated water is similar to control during the experiment period (72 hours).

4.4.2 Discharge treated water - 3-32 psu (Fig. 4)

For the 3-32 psu discharge treated water, growth curves show that the growth at all test concentrations of the discharge treated water is similar to control during the experiment period (72 hours).

4.5 Specific growth rate and coefficient of variation

4.5.1 Discharge treated water - > 32 psu (Table 8, 10)

The mean specific growth rate in the control over the experiment duration was ranged from 1.51 to 2.04 day^{-1} higher than 0.90 day^{-1} as required validity of the experiment. The mean specific growth rate in the each test concentration was ranged from 1.45 to 2.04 day^{-1}. The variation coefficient of specific growth rate (SGR) in the control was ranged from 1.1 to 3.0 % and mean variation coefficient of SGR was 1.8 % less than 7.0 % required validity of the experiment.

4.5.2 Discharge treated water - 3-32 psu (Table 9, 10)

The mean specific growth rate in the control over the experiment duration was ranged from 1.82 to 2.23 day^{-1} higher than 0.90 day^{-1} as required validity of the experiment. The mean specific growth rate in the each test concentration was ranged from 1.81 to 2.62 day^{-1}. The variation coefficient of specific growth rate (SGR) in the control was ranged from 0.4 to 2.1 % and mean variation coefficient of SGR was 1.0 % less than 7.0 % required validity of the experiment.

4.6 Percent inhibition in average specific growth rate

4.6.1 Discharge treated water - > 32 psu (Table 11)

The percent inhibition in average specific growth rate for the each test concentration was ranged from -7.53 to 4.55 %. The percent inhibition in average specific growth rate at the end of the experiment was ranged from -3.30 to 0.85 %.

4.6.2 Discharge treated water - 3-32 psu (Table 12)

The percent inhibition in average specific growth rate for the each test concentration was ranged from -32.66 to 0.07 %. The percent inhibition in average specific growth rate at the end of the experiment was ranged from -2.12 to 0.07 %.

4.7 Plotting of the concentration response curve (Figure 5, 6)

The cell density data on the each test concentration of > 32 and 3-32 psu discharge treated water from the BioVioletTM was plotted on the graph.

4.8 ECx, LOEC and NOEC (Table 13)

For the > 32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 72h-EC50 at the end of the experiment were equal to or higher than 100.00 % discharge treated water.

4.9 Other observation

Any abnormal appearance of the algae during the experiment period was not observed.

5. DISCUSSION AND CONCLUSION

The inhibition growth test of *Skeletonema costatum* on > 32 and 3-32 psu discharge treated water was carried out in a concentration series from 0.00 % (Control) to 100.00 % that were made by the discharge treated water discharge treated water diluted in non-discharge treated water. The growth rate, coefficient variation and test conditions were met with required validity of experiment.

For > 32 psu discharge treated water, growth curves (Fig. 3) based on the cell density (Table 6) calculated from chlorophyll *a* value (Table 4) show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (72 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -7.53 to 4.55 percent (Table 11). Values of NOEC and LOEC calculated by growth rate at the end of the experiment were equal to or higher than 100.00 % discharge treated water. So, 72h-EC50 value was higher than 100.00 % discharge treated water (Table 13).

For 3-32 psu discharge treated water, growth curves (Fig. 4) based on the cell density (Table 7) calculated from chlorophyll *a* value (Table 5) show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (72 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -32.66 to 0.07 (Table 12). Values of NOEC and LOEC calculated by growth rate at the end of the experiment were equal to or higher than 100.00 % discharge treated water. So, 72h-EC50 value was higher than 100.00 % discharge treated water (Table 13).

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have toxic effects on the growth of diatom, *Skeletonema costatum*.

6. REFERENCE

ISO 10253. 2006. International Standard, water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornautum*. 12pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013

TABLES

Table 1. Reference toxicant test result with *Skeletonema costatum* for precision object

Test substance	72h-EC50 (mg/L)	95 % confidence of limit (mg/L)	
		lower	upper
Potassium dichromate	3.4	3.2	3.6

Table 2. For > 32 psu, pH values of test concentration series at the beginning and the end of the experiment

Concentration (%)	Control	6.25	12.5	25	50	100
Initial	7.78	7.74	7.77	7.77	7.74	7.76
Final	9.01	9.06	9.02	8.99	9.01	9.11

Table 3. For 3-32 psu, pH values of test concentration series at the beginning and the end of the experiment

Concentration (%)	Control	6.25	12.5	25	50	100
Initial	7.67	7.65	7.70	7.73	7.70	7.70
Final	9.02	9.17	9.13	9.13	9.16	9.20

Table 4. Chlorophyll *a* (μg/mL) of *Skeletonema costatum* at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	5.67	5.41	5.61	5.56	5.63	6.98	5.30	5.97
24	19.00	18.30	18.50	18.60	18.50	21.20	17.30	19.00
48	163.00	144.00	133.00	146.67	161.00	138.00	123.00	140.67
72	275.00	236.00	254.00	255.00	238.00	286.00	266.00	263.33
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	5.60	5.85	5.51	5.65	6.45	6.40	5.67	6.17
24	20.30	19.70	20.30	20.10	20.00	19.50	19.80	19.77
48	157.00	149.00	132.00	146.00	146.00	140.00	133.00	139.67
72	245.00	258.00	273.00	258.67	286.00	287.00	287.00	286.67
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	5.16	5.49	5.12	5.26	5.23	5.20	5.12	5.18
24	18.50	19.50	17.30	18.43	17.30	20.80	19.00	19.03
48	135.00	146.00	114.00	131.67	119.00	142.00	148.00	136.33
72	260.00	289.00	270.00	273.00	208.00	245.00	266.00	239.67

Table 5. Chlorophyll *a* (μg/mL) of *Skeletonema costatum* at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time	Control				6.25 %			
(h)	1	2	3	Mean	1	2	3	Mean
0	2.95	2.64	2.45	2.68	2.78	2.69	2.96	2.81
24	15.00	13.60	12.60	13.73	18.80	19.90	21.00	19.90
48	120.00	103.00	96.10	106.37	162.00	191.00	173.00	175.33
72	243.00	236.00	243.00	240.67	262.00	274.00	277.00	271.00
Elapsed time	**12.50 %**				**25.00 %**			
(h)	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	2.98	3.26	3.11	3.12	2.67	2.48	2.78	2.64
24	20.40	20.00	19.00	19.80	15.00	22.50	14.90	17.47
48	168.00	129.00	146.00	147.67	164.00	186.00	125.00	158.33
72	264.00	310.00	267.00	280.33	264.00	252.00	269.00	261.67
Elapsed time	**50.00 %**				**100.00 %**			
(h)	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	3.65	2.60	2.98	3.08	2.80	2.49	3.21	2.83
24	23.50	19.80	16.50	19.93	27.10	21.90	25.20	24.73
48	163.00	160.00	145.00	156.00	171.00	147.00	152.00	156.67
72	310.00	262.00	275.00	282.33	258.00	261.00	281.00	266.67

Table 6. Cell density (cells/mL) of *Skeletonema costatum* calculated by equation from linear correlation analysis at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	3,404	3,210	3,359	3,324	3,374	4,414	3,129	3,639
24	15,433	14,725	14,927	15,028	14,927	17,698	13,726	15,450
48	226,587	194,068	175,717	198,791	223,117	184,013	159,361	188,830
72	435,678	359,865	394,496	396,680	363,681	457,570	417,929	413,060
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,351	3,540	3,284	3,392	3,999	3,960	3,404	3,788
24	16,764	16,147	16,764	16,558	16,455	15,942	16,249	16,215
48	216,209	202,527	174,067	197,601	197,443	187,353	175,717	186,838
72	377,101	402,276	431,721	403,700	457,570	459,571	459,571	458,904
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,026	3,269	2,996	3,097	3,077	3,055	2,996	3,043
24	14,927	15,942	13,726	14,865	13,726	17,281	15,433	15,480
48	179,026	197,443	144,921	173,797	152,909	190,704	200,829	181,481
72	406,178	463,577	425,799	431,852	307,312	377,101	417,929	367,447

Table 7. Cell density (cells/mL) of *Skeletonema costatum* calculated by equation from linear correlation analysis at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	3,009	2,631	2,404	2,681	2,801	2,692	3,022	2,838
24	21,531	19,124	17,436	19,364	28,296	30,312	32,351	30,320
48	266,569	221,581	203,749	230,633	383,277	467,789	414,988	422,018
72	626,013	604,260	626,013	618,762	685,716	723,899	733,501	714,372
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,047	3,396	3,208	3,217	2,667	2,439	2,801	2,636
24	31,236	30,496	28,661	30,131	21,531	35,167	21,358	26,019
48	400,520	290,947	337,961	343,143	389,010	453,013	280,066	374,030
72	692,055	840,519	701,582	744,719	692,055	654,176	707,946	684,726
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,894	2,583	3,047	3,174	2,825	2,451	3,333	2,870
24	37,067	30,128	24,163	30,453	44,045	34,036	40,336	39,472
48	386,142	377,559	335,162	366,288	409,190	340,764	354,839	368,264
72	840,519	685,716	727,097	751,111	673,069	682,551	746,336	700,652

Table 8. Specific growth rate of *Skeletonema costatum* at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25%			
	1	2	3	Mean	1	2	3	Mean
24	1.51	1.52	1.49	1.51	1.49	1.39	1.48	1.45
48	2.10	2.05	1.98	2.04	2.10	1.87	1.97	1.98
72	1.62	1.57	1.59	1.59	1.56	1.55	1.63	1.58
Elapsed time (h)	**12.50%**				**25.00%**			
	1	2	3	Mean	1	2	3	Mean
24	1.61	1.52	1.63	1.59	1.41	1.39	1.56	1.46
48	2.08	2.02	1.99	2.03	1.95	1.93	1.97	1.95
72	1.57	1.58	1.63	1.59	1.58	1.58	1.64	1.60
Elapsed time (h)	**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean
24	1.60	1.58	1.52	1.57	1.50	1.73	1.64	1.62
48	2.04	2.05	1.94	2.01	1.95	2.07	2.10	2.04
72	1.63	1.65	1.65	1.65	1.53	1.61	1.65	1.60

Table 9. Specific growth rate of *Skeletonema costatum* at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25%			
	1	2	3	Mean	1	2	3	Mean
24	1.97	1.98	1.98	1.98	2.31	2.42	2.37	2.37
48	2.24	2.22	2.22	2.23	2.46	2.58	2.46	2.50
72	1.78	1.81	1.85	1.82	1.83	1.86	1.83	1.84
Elapsed time (h)	**12.50%**				**25.00%**			
	1	2	3	Mean	1	2	3	Mean
24	2.33	2.19	2.19	2.24	2.09	2.67	2.03	2.26
48	2.44	2.23	2.33	2.33	2.49	2.61	2.30	2.47
72	1.81	1.84	1.80	1.81	1.85	1.86	1.84	1.85
Elapsed time (h)	**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean
24	2.25	2.46	2.07	2.26	2.75	2.63	2.49	2.62
48	2.30	2.49	2.35	2.38	2.49	2.47	2.33	2.43
72	1.79	1.86	1.83	1.83	1.82	1.88	1.80	1.83

Table 10. Coefficient of variation of specific growth rate in the control during the experiment period

Elapsed time (h)	>32 psu	3-32 psu
24	1.1	0.4
48	3.0	0.6
72	1.4	2.1
Mean	1.8	1.0

Table 11. Percent inhibition in average specific growth rate of *Skeletonema costatum* at the each test concentration of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Concentration (%)				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	3.80	-5.11	3.45	-3.89	-7.53
48	3.30	0.60	4.55	1.61	0.10
72	0.85	0.02	-0.43	-3.30	-0.14

Table 12. Percent inhibition in average specific growth rate of *Skeletonema costatum* at the each test concentration of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Concentration (%)				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	-19.76	-13.14	-14.42	-14.29	-32.66
48	-12.29	-4.71	-10.89	-6.93	-9.14
72	-1.53	0.07	-2.12	-0.58	-1.08

Table 13. NOEC, LOEC, 72h-EC50 value calculated from mean response cell density at the end of the experiment

Test substance	NOEC (%)	LOEC (%)	72h-EC50 (%)	95 % confidence of limit (%)	
				lower	upper
> 32 psu ballast water	100.00	>100.00	>100.00	-	-
3-32 psu ballast water	100.00	>100.00	>100.00	-	-

FIGURES

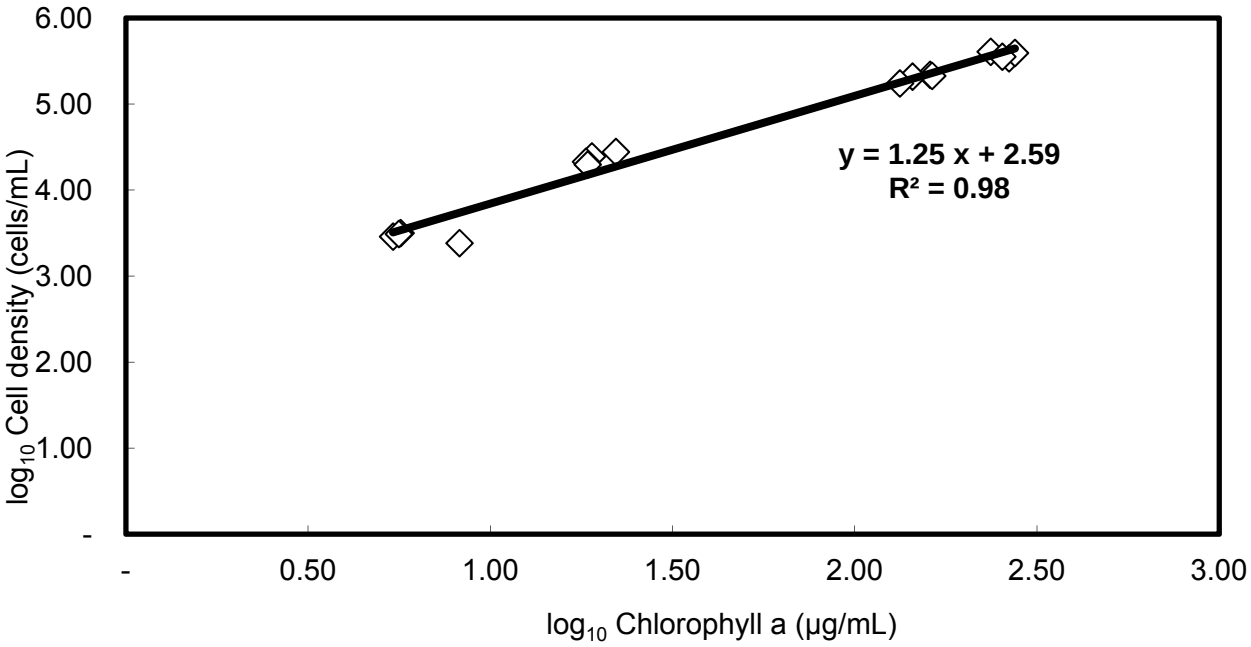

Fig. 1. Cell density in the control as function of chlorophyll *a* concentration (> 32 psu ballast water, n=16, P<0.001).

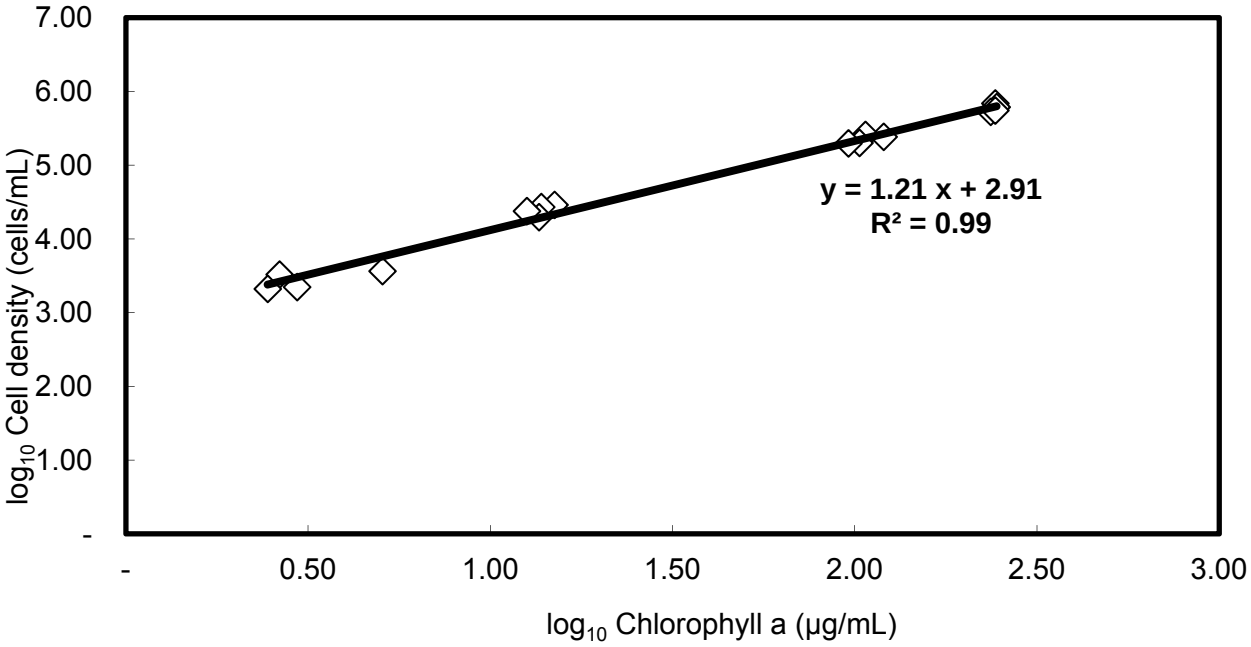

Fig. 2. Cell density in the control as function of chlorophyll *a* concentration (3-32 psu ballast water, n=16, P<0.001).

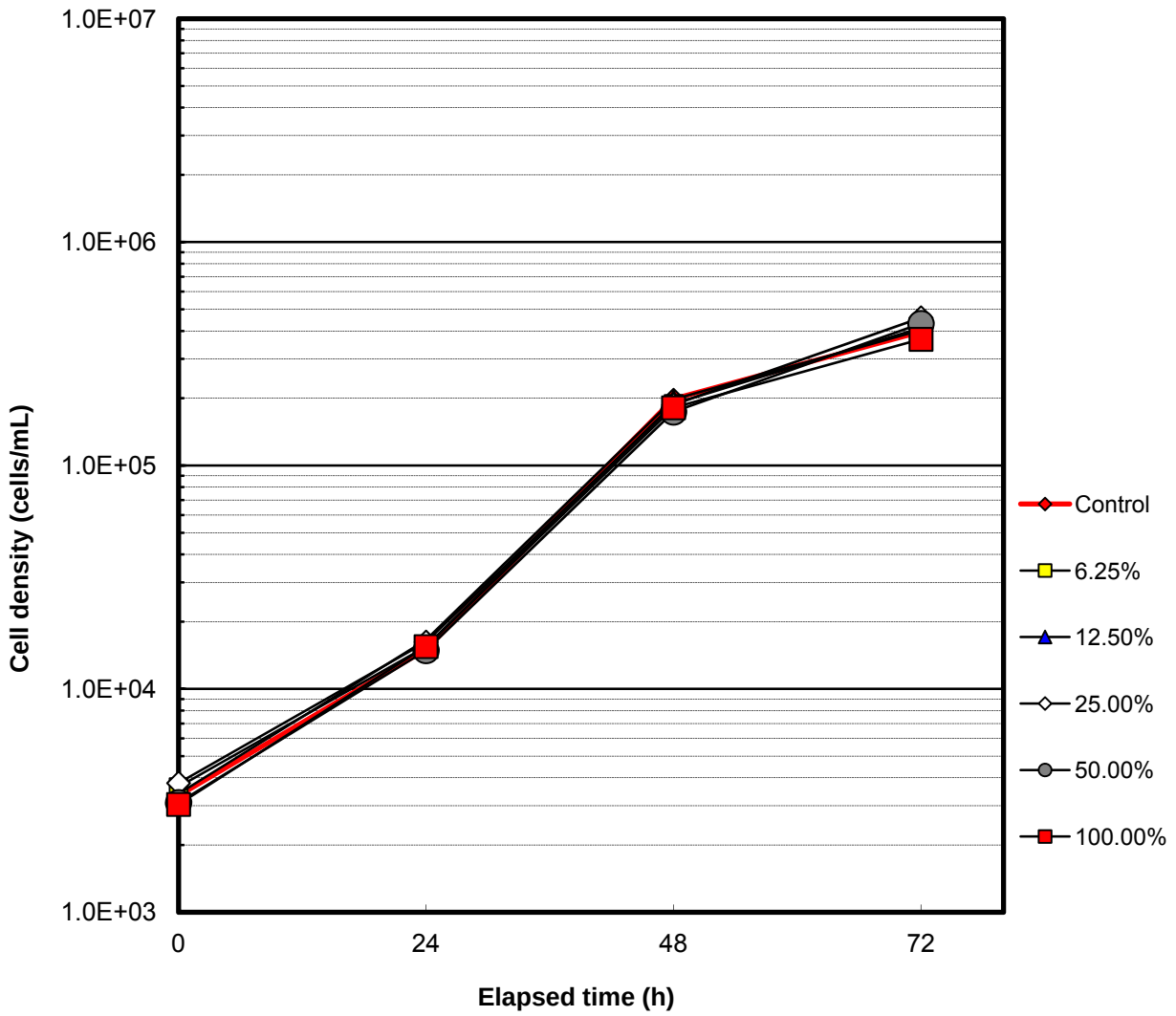

Fig. 3. Growth curves of *Skeletonema costatum* at various concentrations of > 32 psu discharge treated water. The data of cell density was transformed to log value.

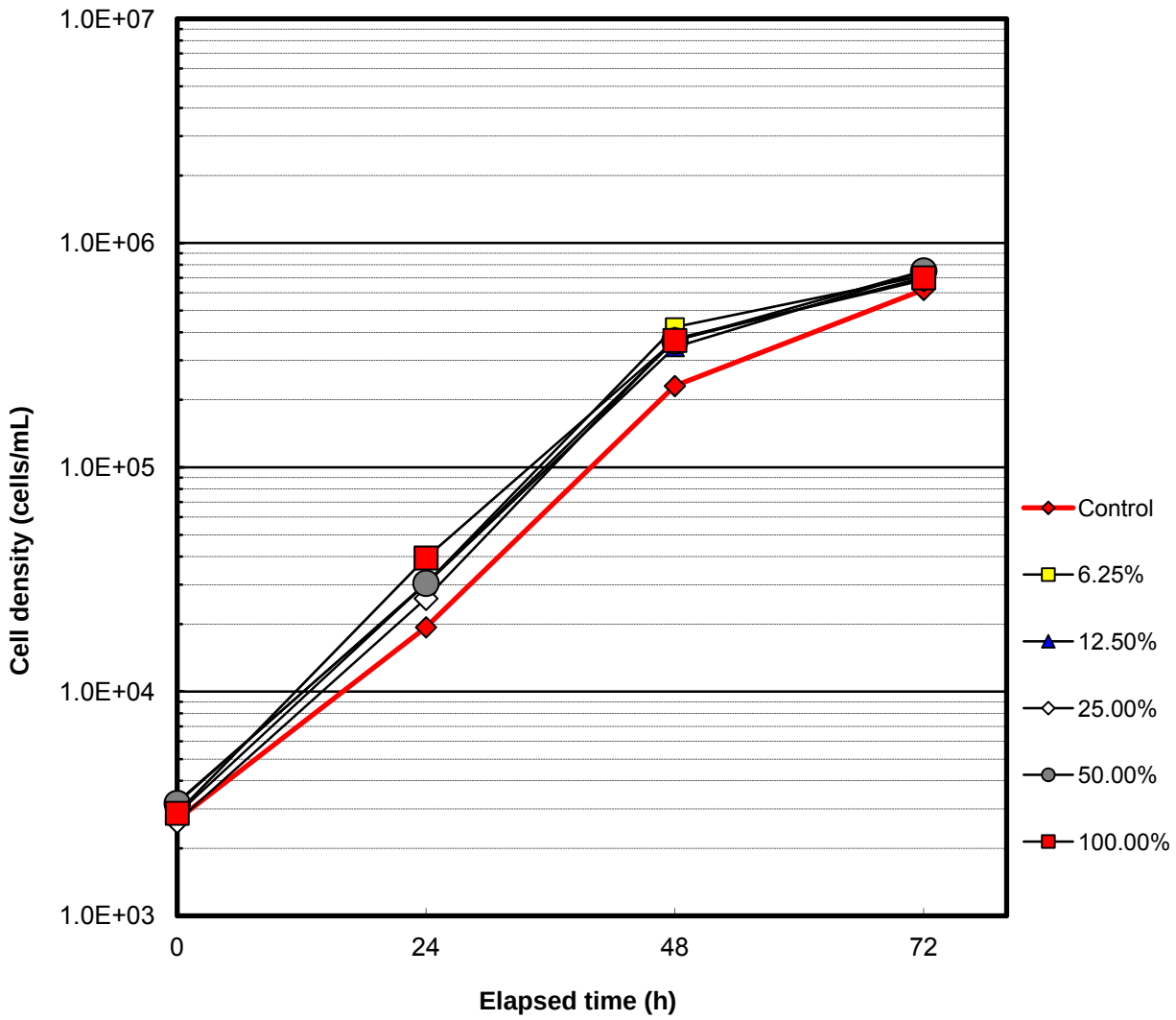

Fig. 4. Growth curves of *Skeletonema costatum* at various concentrations of 3-32 psu discharge treated water. The data of cell density was transformed to log value.

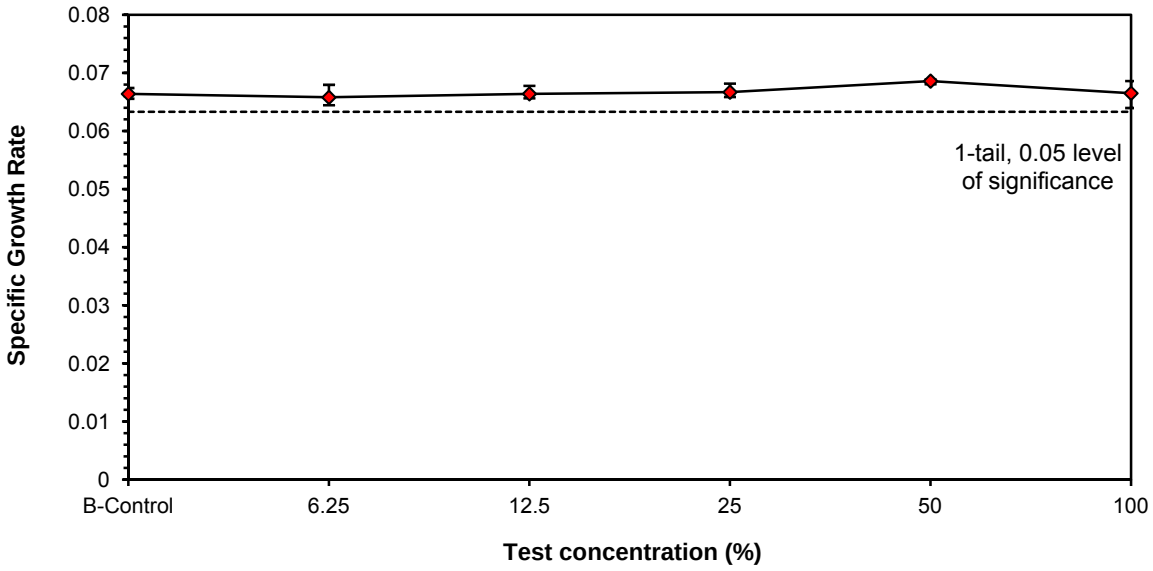

Fig. 5. Plot of the concentration-response data at the end of the experiment with *Skeletonema costatum* in > 32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

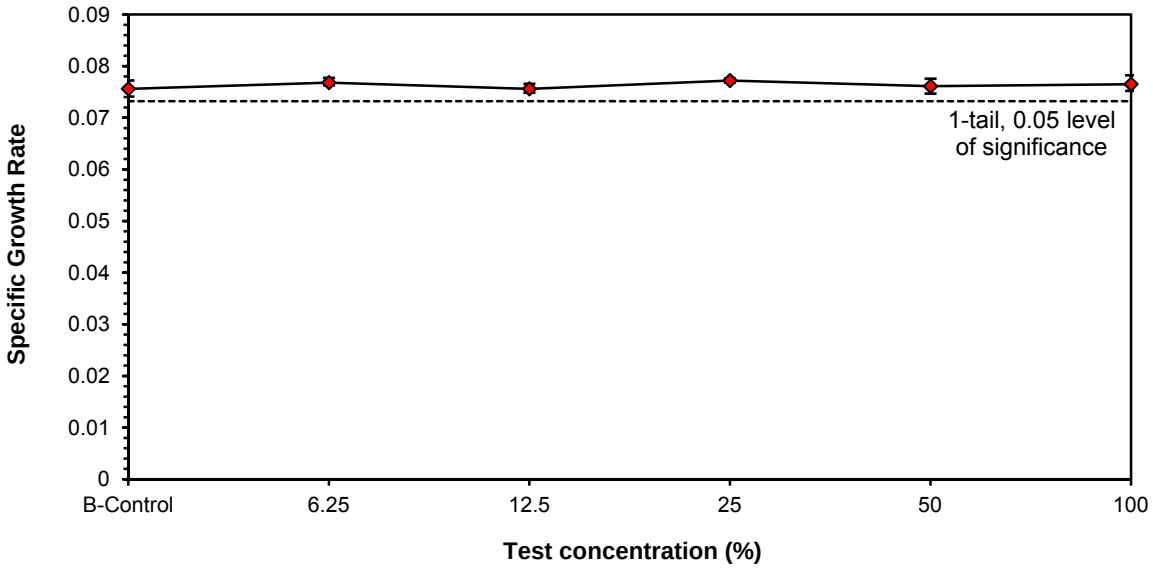

Fig. 6. Plot of the concentration-response data at the end of the experiment with *Skeletonema costatum* in 3-32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

ANNEX 1

Preparation of test substance and procedures of the experiment

Fig. 1. For aquatic toxicity test, collection of the control and treated ballasting water from the BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

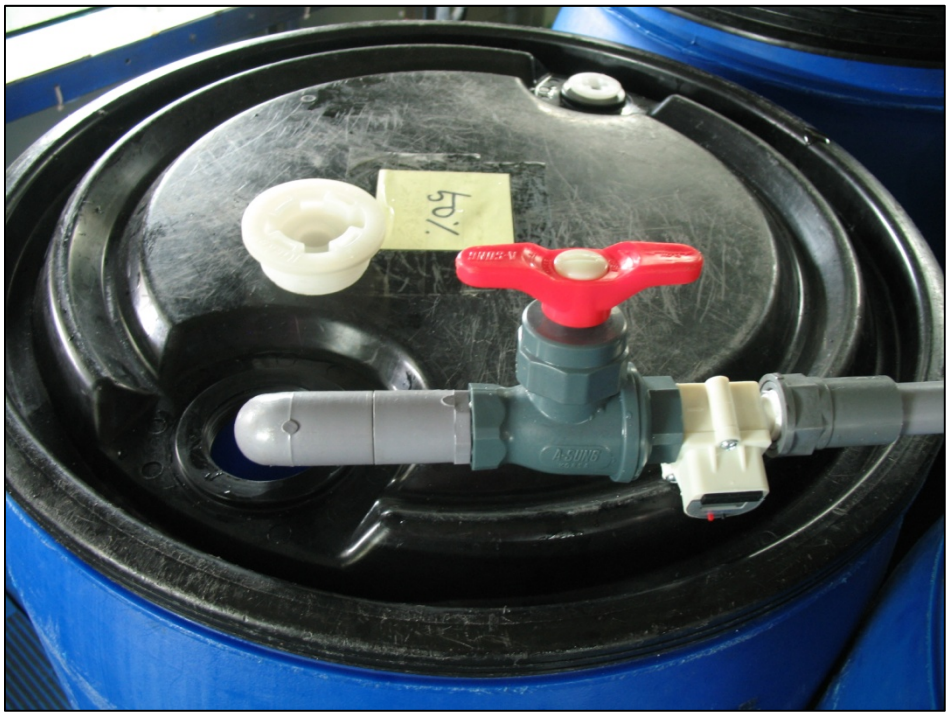

Fig. 3. Preparation of test concentration diluted with non-treated (control) seawater on treated seawater through flow-meter.

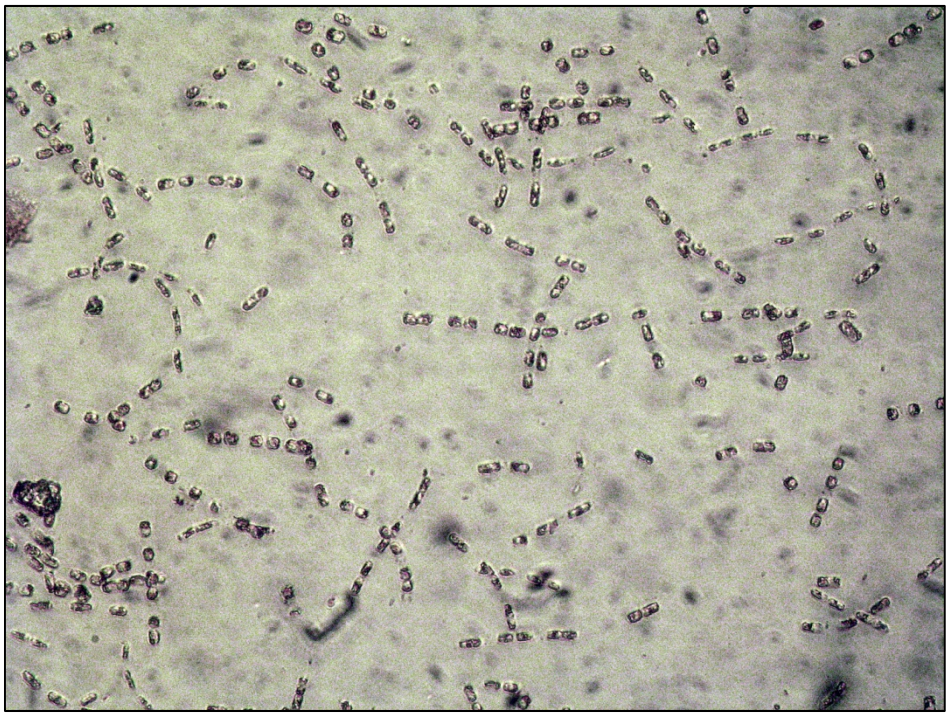

Fig. 4. Test organisms in this study: Aquatic plant (Diatom), *Skeletonema costatum*.

Fig. 5. For the growth inhibition test using the diatom, *Skeletonema costatum*, final preparation of test substance was filtered by 0.45 μm membrane filter.

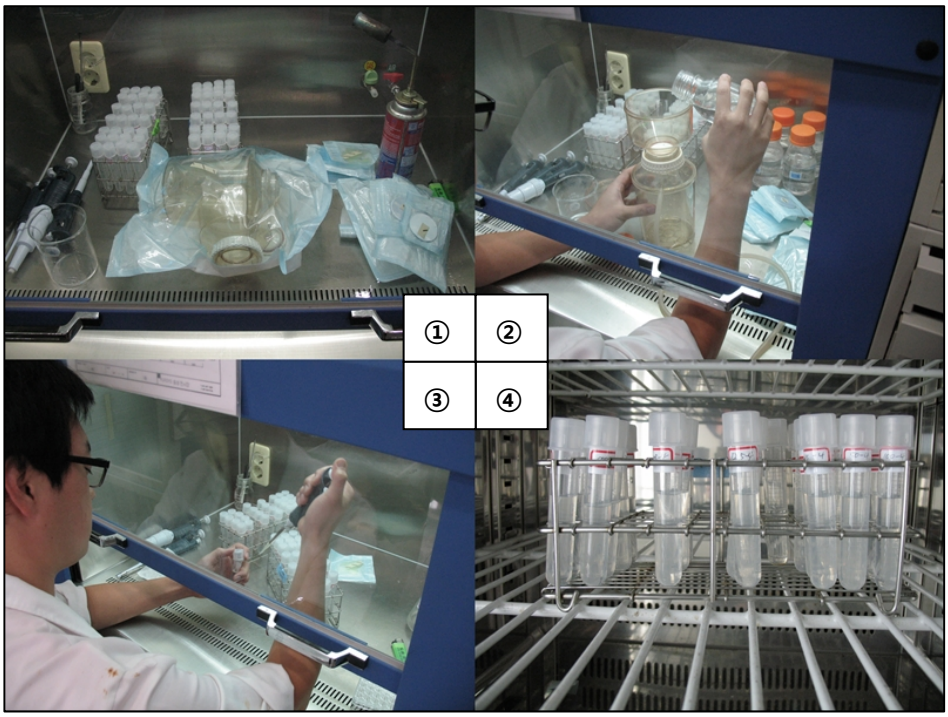

Fig. 6. The inoculation procedures of *Skeletonema costatum* (① Preparation of test apparatus; ② Sterilizing medium by filter (0.2 μm membrane filter); ③ Inoculation; ④ Incubation at 22 ˚C).

Fig. 7. The extraction of chlorophyll *a* at the each test concentration (① Collecting of the samples; ② Adding the aqueous acetone solution; ③ Centrifuging the extract after storage for a 24 hours in the 4˚C cold and dark room; ④ Estimating the chlorophyll *a*).

2.1.2 ANNEX 2

Skeletonema costatum, Growth Inhibition Test - 96 hours

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FINAL REPORT

Aquatic Toxicity For > 32 & 3-32 psu discharge treated water of BioViolet™

- *Skeletonema costatum*, Growth Inhibition Test – 96 hours

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GLP STATEMENT AND SIGN

Study title: Algae growth inhibition test-96h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward *Skeletonema costatum*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

All procedure of this study was carried out following GLP regulation:

- "OECD Principles of Good Laboratory Practice"
  Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

This study was conducted on the basis of 'the Study Plan' complied and the procedure described in 'the Study Plan' for this report was carried out under 'the Responsibility of Study Director'. This report was built on the basis of raw data from the study.

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Study Director Myung-Baek Shon (signature)

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Test Facility Manager Min Ho Son (signature)

GUARANTEE OF ASSURANCE

Study title: Algae growth inhibition test-96h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward *Skeletonema costatum*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

Quality assurance personnel of Marine Eco-technology Institute Co., Ltd., inspected as follows. Inspection was carried out on the basis of the Standard Operation Procedure and each inspection results were reported to Study Director and Test Facility Manager.

Types of inspection and the date inspection results were reported to the Test Facility Management and the Study Director as follow:

Type of Inspections	Date of Inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011. 02. 28	2011. 02. 28
Test organisms	2011. 10. 08 / 10. 29	2011. 10. 10 / 10. 31
Storage of Test substance	2011. 10. 10 / 10. 31	2011. 10. 10 / 10. 31
Preparation of Test substance	2011. 10. 11 / 11. 01	2011. 10. 11 / 11. 01
Exposure	2011. 10. 11 / 11. 01	2011. 10. 11 / 11. 01
Observation	2011. 10. 12 / 11. 02	2011. 10. 12 / 11. 02
Record of raw data	2011. 10. 12 / 11. 02	2011. 10. 12 / 11. 02
Draft of final report	2011. 12. 23	2011. 12. 23
Final report	2011. 12. 30	2011. 12. 30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Jin Hee Kim (signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

Page

1. SUMMARY

The purpose of this study was to assess the chronic toxic effects of the > 32 and 3-32 psu treated ballas water from the BioVioletTM on the growth of diatom, *Skeletonema costatum*.

The test substances were divided into two different salinities, the one was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. Diatom, *Skeletonema costatum* inoculated 3,536±448 cells/mL (mean±S.D) at > 32 psu and 4,289±395 cells/mL (mean±S.D) at 3-32 psu on the exponential phase was incubated at the various test concentrations (0.00 % (non-discharge treated water), 6.25, 12.50, 25.00, 50.00 and 100.00 % (discharge treated water)) for 96 hours. The cell density was counted daily by measuring chlorophyll *a* with 90% acetone using Tuner Designs AU-10 including the control. To obtain the regression equation between chlorophyll *a* and cell density, cell count in the control was done by manual cell counting used the microscope with counting chamber. The raw data of growth from this experiment was used for calculation of inhibition of growth rate and values of 96h-EC50, LOEC and NOEC.

The inhibition growth test of *Skeletonema costatum* on > 32 and 3-32 psu discharge treated water was carried out in a concentration series from 0.00 % (Control) to 100.00 % that were made by the discharge treated water diluted in non-discharge treated water.

For > 32 psu and 3-32 psu discharge treated water, growth curves based on the cell density calculated from chlorophyll *a* value show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (96 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -19.05 to 7.53 percent in >32 psu and from -26.48 to 5.47 percent in 3-32 psu discharge treated water.

For >32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 96h-EC50 calculated from growth rate were equal to or higher than 100.00 % discharge treated water at the end of the experiment.

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have toxic effects on the growth of diatom, *Skeletonema costatum*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 10. 29
Pre-culture for test	2011. 10. 29
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01
Evaluation of cell density	2011. 11. 01 ~ 11. 05
Evaluation of chlorophyll *a*	2011. 11. 02 ~ 11. 06
Experimental completion	2011. 11. 05
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 10. 08
Pre-culture for test	2011. 10. 08
Collect of sample	2011. 10. 10
Experimental start	2011. 10. 11
Exposure date	2011. 10. 11
Evaluation of cell density	2011. 10. 11 ~ 10. 15
Evaluation of chlorophyll *a*	2011. 10. 12 ~ 10. 16
Experimental completion	2011. 10. 15
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For satisfactory correlation with biomass, chlorophyll *a* and cell density in the control were analyzed by linear correlation analysis with Excel program and the greatest R^2 value was yielded. Cell density was calculated by equation from the linear correlation analysis.

For > 32 psu and 3-32 psu discharge treated water, Shapiro-Wilk's Test and Bartlett's Test were respectively used to normality and homogeneity of variance of specific growth rate. Dunnett's Test was used to determine the NOEC and LOEC. Differences in all tests were considered to be significant at α=0.05. TOXCALC 5.0 program (Tidepool scientific software, USA) was used for above statistic analysis.

4. RESULTS

4.1 pH values (Table 1, 2)

For >32 psu discharge treated water, the initial pH value was ranged from 7.63 to 7.87 and the final one was ranged from 9.04 to 9.16. For 3-32 psu discharge treated water, the initial pH value was ranged from 7.65 to 7.73 and the final one was ranged from 8.94 to 9.16.

4.2 Biomass

4.2.1 Discharge treated water - > 32 psu (Table 3, 5, Figure 1)

The values of the chlorophyll *a* and cell density had functional relationship indicated by the equation Y=1.31X+2.53 (R^2=0.96, n=16, P<0.001). Therefore, cell density in the each test concentration including control was calculated from the equation. The initial cell density in the test concentrations was ranged from 2,346 to 3,935 cells/mL (mean±S.D: 3,536±448 cells/mL). The mean cell density in the control at the end of the experiment was 578,895 cells/mL. The mean cell densities in the each test concentration at the end of the experiment, 6.25, 12.50, 25.00, 50.00 and 100.00 % were ranged from 471,230 to 535,875 cells/mL.

4.2.2 Discharge treated water - 3-32 psu (Table 4, 6, Figure 2)

The values of the chlorophyll *a* and cell density had functional relationship indicated by the equation Y=0.91X+3.51 (R^2=0.98, n=16, P<0.001). Therefore, cell density in the each test concentration including control was calculated from the equation. The initial cell density in the test concentrations was ranged from 3,558 to 5,104 cells/mL (mean±S.D: 4,289±395 cells/mL). The mean cell density in the control at the end of the experiment was 539,687 cells/mL. The mean cell densities in the each test concentration at the end of the experiment, 6.25, 12.50, 25.00, 50.00 and 100.00 % were 544,456 to 593,280cells/mL.

4.3 Growth curve

4.3.1 Discharge treated water - > 32 psu (Fig. 3)

For the >32 psu discharge treated water, growth curves showed that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (96 hours).

4.3.2 Discharge treated water - 3-32 psu (Fig. 4)

For the 3-32 psu discharge treated water, growth curves showed that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (96 hours).

4.4 Specific growth rate and coefficient of variation

4.4.1 Discharge treated water - > 32 psu (Table 7, 9)

The mean specific growth rate in the control over the experiment duration was ranged from 1.25 to 1.94 day^{-1} higher than 0.9 day^{-1} as required validity of the experiment. The mean specific growth rate in the each test concentration was ranged from 1.20 to 2.13 day^{-1}. The variation coefficient of specific growth rate (SGR) in the control was ranged from 1.1 to 4.7 % and mean variation coefficient of SGR was 3.3 % less than 7 % required validity of the experiment.

4.4.2 Discharge treated water - 3-32 psu (Table 8, 9)

The mean specific growth rate in the control over the experiment duration was ranged from 1.24 to 1.99 day^{-1} higher than 0.9 day^{-1} as required validity of the experiment. The mean specific growth rate in the each test concentration was ranged from 1.20 to 2.51 day^{-1}. The variation coefficient of specific growth rate (SGR) in the control was ranged from 0.4 to 6.4 % and mean variation coefficient of SGR was 2.3 % less than 7 % required validity of the experiment.

4.5 Percent inhibition of average specific growth rate

4.5.1 Discharge treated water - > 32 psu (Table 10)

The percent inhibition in average specific growth rate for the each test concentration was ranged from -19.05 to 7.53 %. The percent inhibition in average specific growth rate at the end of the experiment was ranged from -2.08 to 3.67 %.

4.5.2 Discharge treated water - 3-32 psu (Table 11)

The percent inhibition in average specific growth rate for the each test concentration was ranged from -26.48 to 5.47 %. The percent inhibition in average specific growth rate at the end of the experiment was ranged from 1.39 to 3.19 %.

4.6 Plotting of the concentration response curve (Figure 5, 6)

The cell density data on the each test concentration of > 32 and 3-32 psu discharge treated water by the BioViolet™ was plotted on the graph.

4.7 ECx, LOEC and NOEC (Table 12)

For the > 32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 96h-EC50 at the end of the experiment were equal to or higher than 100.00 % discharge treated water.

4.8 Other observation

Any abnormal appearance of the algae during the experiment period was not observed.

5. DISCUSSION AND CONCLUSION

The inhibition growth test of *Skeletonema costatum* on > 32 and 3-32 psu discharge treated water was carried out in a concentration series from 0.00 % (Control) to 100.00 % that were made by the discharge treated water diluted in non-discharge treated water.

For > 32 psu discharge treated water, growth curves (Fig. 3) based on the cell density (Table 5) calculated from chlorophyll *a* value (Table 3) show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (96 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -19.05 to 7.53 percent (Table 10). Values of NOEC and LOEC calculated by growth rate at the end of the experiment were equal to or higher than 100.00 % discharge treated water. So, 96h-EC50 value was higher than 100.00 % discharge treated water (Table 12).

For 3-32 psu discharge treated water, growth curves (Fig. 4) based on the cell density (Table 6) calculated from chlorophyll *a* value (Table 4) show that the growth at all test concentrations of the discharge treated water was similar to control during the experiment period (96 hours). And percent inhibitions in specific average growth rate at all test concentrations were ranged from -26.48 to 5.47 percent (Table 11). Values of NOEC and LOEC calculated by growth rate at the end of the experiment were equal to or higher than 100.00 % discharge treated water. At the end of the experiment, 96h-EC50 value from point estimation using linear interpolation was higher than 100.00 % discharge treated water. So, 96h-EC50 value was higher than 100.00 % discharge treated water (Table 12).

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioViolet™ did not have toxic effects on the growth of diatom, *Skeletonema costatum*.

6. REFERENCE

ISO 10253. 2006. International Standard, water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornautum*. 12pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

TABLES

Table 1. For > 32 psu discharge treated water, pH values of test concentration series at the beginning and the end of the experiment

Concentration (%)	Control	6.25	12.5	25	50	100
Initial	7.75	7.72	7.65	7.63	7.73	7.87
Final	9.04	9.16	9.10	9.13	9.08	9.16

Table 2. For 3-32 psu discharge treated water, pH values of test concentration series at the beginning and the end of the experiment

Concentration (%)	Control	6.25	12.5	25	50	100
Initial	7.67	7.65	7.70	7.73	7.70	7.70
Final	8.94	9.05	9.09	9.10	9.15	9.16

Table 3. Chlorophyll *a* (μg /mL) of *Skeletonema costatum* at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	6.50	6.31	6.40	6.40	6.35	6.45	6.24	6.35
24	20.90	19.50	18.90	19.77	18.60	18.90	16.80	18.10
48	124.00	126.00	120.00	123.33	127.00	133.00	107.00	122.33
72	242.00	309.00	291.00	280.67	209.00	211.00	210.00	210.00
96	240.30	323.00	315.00	292.77	231.00	248.00	273.00	250.67
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	6.07	5.06	6.09	5.74	4.38	6.25	6.01	5.55
24	22.30	20.80	20.30	21.13	22.00	21.10	19.80	20.97
48	165.00	148.00	130.00	147.67	142.00	135.00	121.00	132.67
72	213.00	224.00	251.00	229.33	234.00	262.00	259.00	251.67
96	255.00	259.00	268.00	260.67	272.00	257.00	278.00	269.00
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	6.42	6.43	6.23	6.36	5.98	5.27	5.24	5.50
24	17.80	19.00	17.30	18.03	19.80	19.50	21.00	20.10
48	131.00	121.00	116.00	122.67	129.00	131.00	112.00	124.00
72	219.00	255.00	238.00	237.33	200.00	226.00	245.00	223.67
96	264.0	280.0	286.00	276.67	251.00	263.00	258.00	257.33

Table 4. Chlorophyll *a* (μg /mL) of *Skeletonema costatum* at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	1.11	1.20	1.20	1.17	1.40	1.41	1.39	1.40
24	11.60	9.93	9.75	10.43	12.30	15.10	13.70	13.70
48	77.30	74.20	78.80	76.77	104.00	132.00	130.00	122.00
72	299.00	309.00	314.00	307.33	354.00	358.00	362.00	358.00
96	267.00	268.00	295.00	276.67	307.00	324.00	290.00	307.00
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	1.35	1.27	1.65	1.42	1.43	1.42	1.36	1.40
24	15.00	13.90	12.50	13.80	16.50	17.70	16.20	16.80
48	123.00	142.00	127.00	130.67	180.00	157.00	160.00	165.67
72	275.00	290.00	303.00	289.33	269.00	314.00	315.00	299.33
96	294.00	288.00	286.00	289.33	290.00	295.00	283.00	289.33
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	1.44	1.23	1.60	1.42	1.25	1.36	1.47	1.36
24	18.50	15.60	18.90	17.67	20.00	21.10	23.60	21.57
48	162.00	177.00	171.00	170.00	182.00	176.00	184.00	180.67
72	247.00	292.00	286.00	275.00	299.00	283.00	300.00	294.00
96	279.0	279.0	285.00	281.00	271.00	286.00	281.00	279.33

Table 5. Cell density (cells/mL) of *Skeletonema costatum* calculated by equation from linear correlation analysis at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	3,935	3,785	3,856	3,858	3,816	3,895	3,730	3,814
24	18,172	16,594	15,928	16,898	15,598	15,928	13,651	15,059
48	187,233	191,199	179,361	185,931	193,189	205,232	154,345	184,256
72	449,571	619,221	572,400	547,064	371,015	375,673	373,342	373,344
96	445,438	656,229	635,019	578,895	422,992	464,228	526,469	471,230
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,597	2,834	3,613	3,348	2,346	3,738	3,551	3,211
24	19,782	18,058	17,491	18,444	19,435	18,400	16,929	18,254
48	272,210	236,071	199,189	235,823	223,613	209,285	181,321	204,740
72	380,345	406,279	471,599	419,408	430,202	498,855	491,386	473,481
96	481,468	491,386	513,874	495,576	523,944	486,421	539,136	516,501
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	3,871	3,879	3,722	3,824	3,528	2,989	2,967	3,161
24	14,725	16,039	14,185	14,983	16,929	16,594	18,286	17,269
48	201,199	181,321	171,569	184,697	197,184	201,199	163,861	187,415
72	394,441	481,468	439,861	438,590	350,227	411,038	456,886	406,050
96	503,850	544,223	559,551	535,875	471,599	501,351	488,902	487,284

Table 6. Cell density (cells/mL) of *Skeletonema costatum* calculated by equation from linear correlation analysis at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %			
	1	2	3	Mean	1	2	3	Mean
0	3,558	3,820	3,820	3,733	4,395	4,424	4,367	4,395
24	30,106	26,135	25,704	27,315	31,755	38,271	35,028	35,018
48	169,139	162,955	172,123	168,072	221,565	275,247	271,449	256,087
72	579,243	596,846	605,628	593,906	675,450	682,391	689,326	682,389
96	522,547	524,328	572,187	539,687	593,329	623,155	563,355	593,280
Elapsed time (h)	**12.50 %**				**25.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	4,252	4,022	5,104	4,459	4,481	4,452	4,281	4,405
24	38,040	35,493	32,225	35,253	41,487	44,224	40,800	42,170
48	258,115	294,159	265,743	272,672	365,004	322,306	327,906	338,405
72	536,776	563,355	586,290	562,140	526,108	605,628	607,383	579,706
96	570,422	559,818	556,280	562,173	563,355	572,187	550,967	562,170
Elapsed time (h)	**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean
0	4,509	3,907	4,963	4,460	3,964	4,281	4,595	4,280
24	46,039	39,422	46,944	44,135	49,424	51,892	57,458	52,924
48	331,634	359,464	348,359	346,485	368,693	357,616	372,378	366,229
72	486,804	566,889	556,280	536,658	579,243	550,967	581,006	570,405
96	543,876	543,876	554,509	547,420	529,666	556,280	547,423	544,456

Table 7. Specific growth rate of *Skeletonema costatum* at various concentrations of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25%			
	1	2	3	Mean	1	2	3	Mean
24	1.53	1.48	1.42	1.48	1.41	1.41	1.30	1.37
48	1.93	1.96	1.92	1.94	1.96	1.98	1.86	1.94
72	1.58	1.70	1.67	1.65	1.53	1.52	1.54	1.53
96	1.18	1.29	1.28	1.25	1.18	1.20	1.24	1.20
Elapsed time (h)	**12.50%**				**25.00%**			
	1	2	3	Mean	1	2	3	Mean
24	1.70	1.85	1.58	1.71	2.11	1.59	1.56	1.76
48	2.16	2.21	2.00	2.13	2.28	2.01	1.97	2.09
72	1.55	1.66	1.62	1.61	1.74	1.63	1.64	1.67
96	1.22	1.29	1.24	1.25	1.35	1.22	1.26	1.28
Elapsed time (h)	**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean
24	1.34	1.42	1.34	1.36	1.57	1.71	1.82	1.70
48	1.98	1.92	1.92	1.94	2.01	2.10	2.01	2.04
72	1.54	1.61	1.59	1.58	1.53	1.64	1.68	1.62
96	1.22	1.24	1.25	1.24	1.22	1.28	1.28	1.26

Table 8. Specific growth rate of *Skeletonema costatum* at various concentrations of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25%			
	1	2	3	Mean	1	2	3	Mean
24	2.14	1.92	1.91	1.99	1.98	2.16	2.08	2.07
48	1.93	1.88	1.90	1.90	1.96	2.07	2.06	2.03
72	1.70	1.68	1.69	1.69	1.68	1.68	1.69	1.68
96	1.25	1.23	1.25	1.24	1.23	1.24	1.21	1.23
Elapsed time (h)	**12.50%**				**25.00%**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
24	2.19	2.18	1.84	2.07	2.23	2.30	2.25	2.26
48	2.05	2.15	1.98	2.06	2.20	2.14	2.17	2.17
72	1.61	1.65	1.58	1.61	1.59	1.64	1.65	1.63
96	1.22	1.23	1.17	1.21	1.21	1.21	1.21	1.21
Elapsed time (h)	**50.00%**				**100.00%**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
24	2.32	2.31	2.25	2.29	2.52	2.50	2.53	2.51
48	2.15	2.26	2.13	2.18	2.27	2.21	2.20	2.23
72	1.56	1.66	1.57	1.60	1.66	1.62	1.61	1.63
96	1.20	1.23	1.18	1.20	1.22	1.22	1.20	1.21

Table 9. Coefficient of variation of specific growth rate in the control during the experiment period

Elapsed time (h)	>32 psu	3-32 psu
24	3.8	6.4
48	1.1	1.4
72	3.8	0.4
96	4.7	0.9
Mean	3.3	2.3

Table 10. Percent inhibition in average specific growth rate of *Skeletonema costatum* at the each test concentration of > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Concentration (%)				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	7.07	-15.97	-19.05	7.53	-15.23
48	0.11	-9.75	-7.66	-0.01	-5.33
72	7.31	2.28	-1.34	4.17	1.87
96	3.67	-0.14	-2.08	1.09	-0.89

Table 11. Percent inhibition in average specific growth rate of *Skeletonema costatum* at the each test concentration of 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Concentration (%)				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	-4.23	-4.13	-13.60	-15.37	-26.48
48	-6.64	-8.13	-13.99	-14.43	-16.90
72	0.49	4.51	3.79	5.47	3.47
96	1.39	2.64	2.50	3.19	2.53

Table 12. NOEC, LOEC, 96h-EC50 value calculated from mean response cell density at the end of the experiment

Test substance	NOEC (%)	LOEC (%)	96h-EC50 (%)	95 % confidence of limit (%)	
				lower	upper
> 32 psu ballast water	100.00	>100.00	>100.00	-	-
3-32 psu ballast water	100.00	>100.00	>100.00	-	-

FIGURES

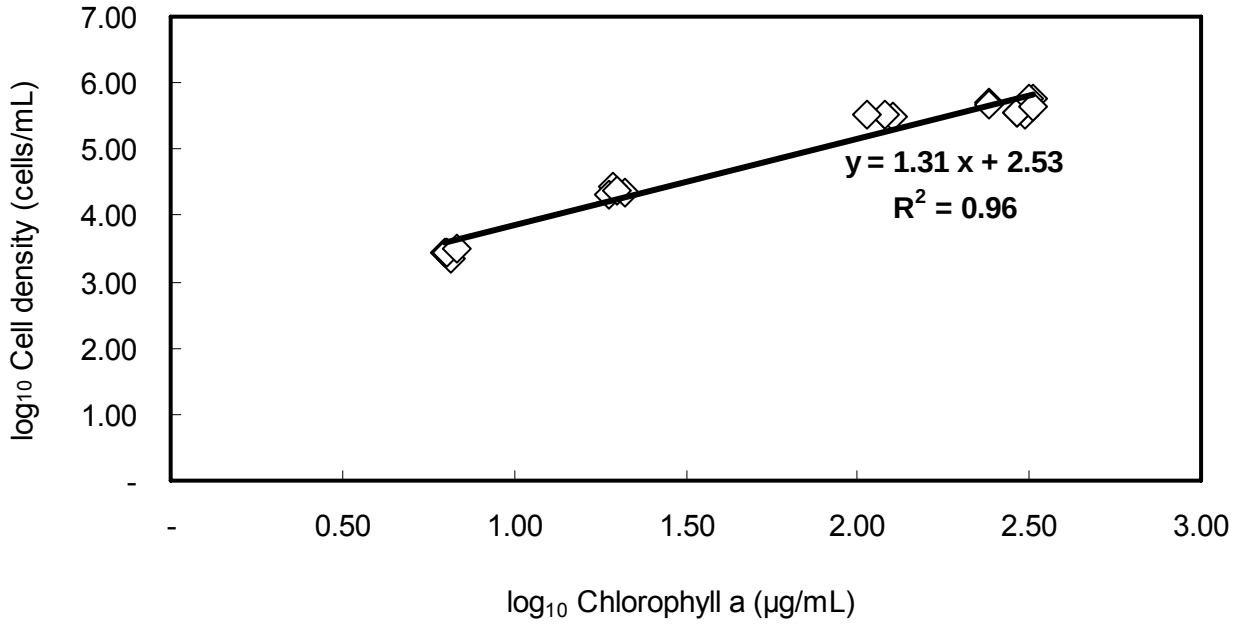

Fig. 1. Cell density in the control as function of chlorophyll *a* concentration (> 32 psu ballast water, n=16, P<0.001).

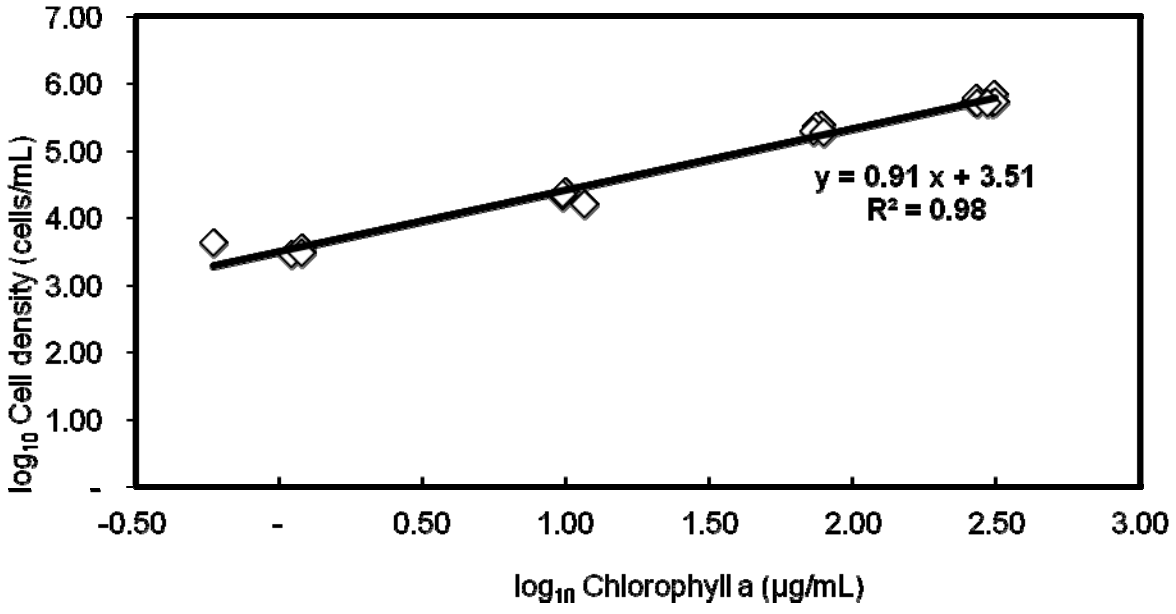

Fig. 2. Cell density in the control as function of chlorophyll *a* concentration (3-32 psu ballast water, n=16, P<0.001).

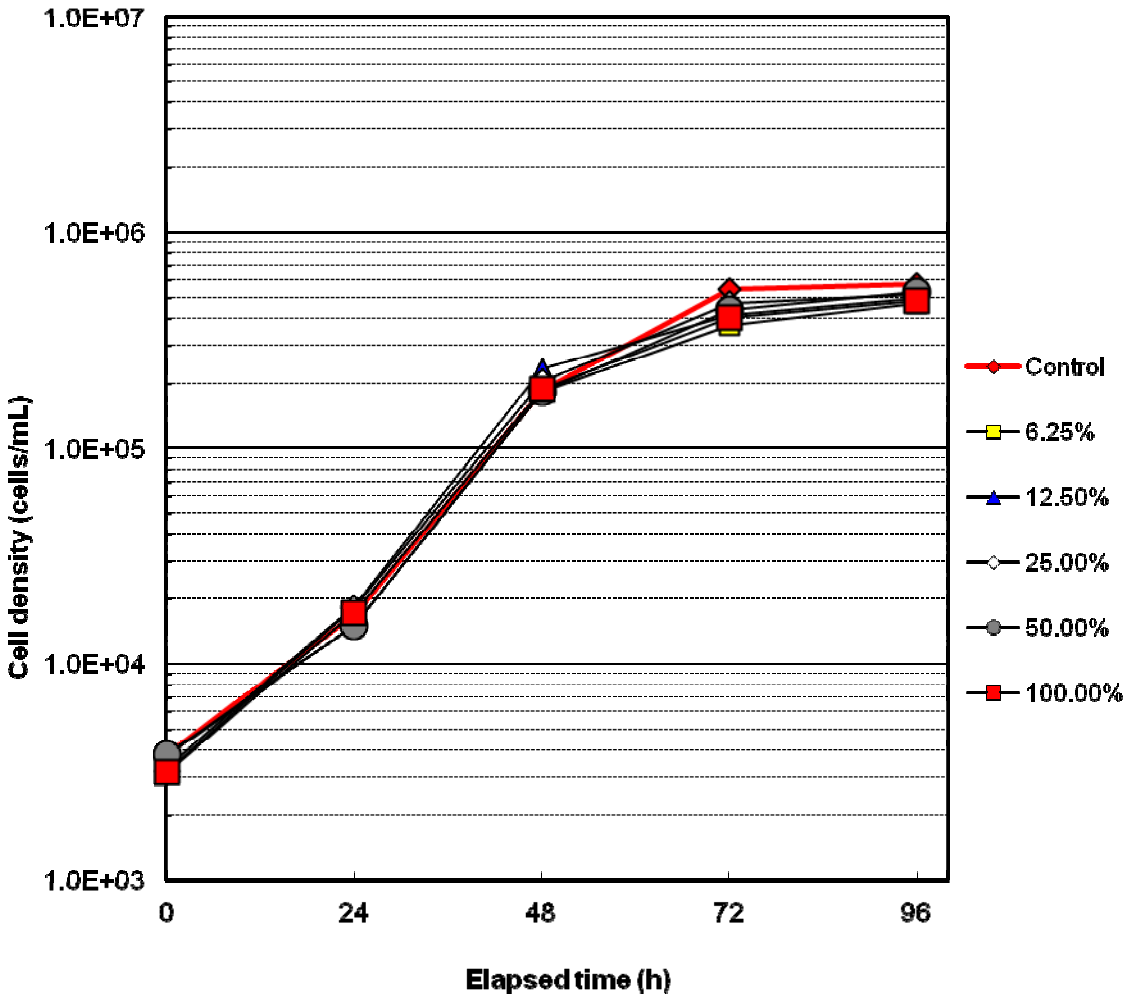

Fig. 3. Growth curves of *Skeletonema costatum* at various concentrations of treated > 32 psu ballast water. The data of cell density was transformed to log value.

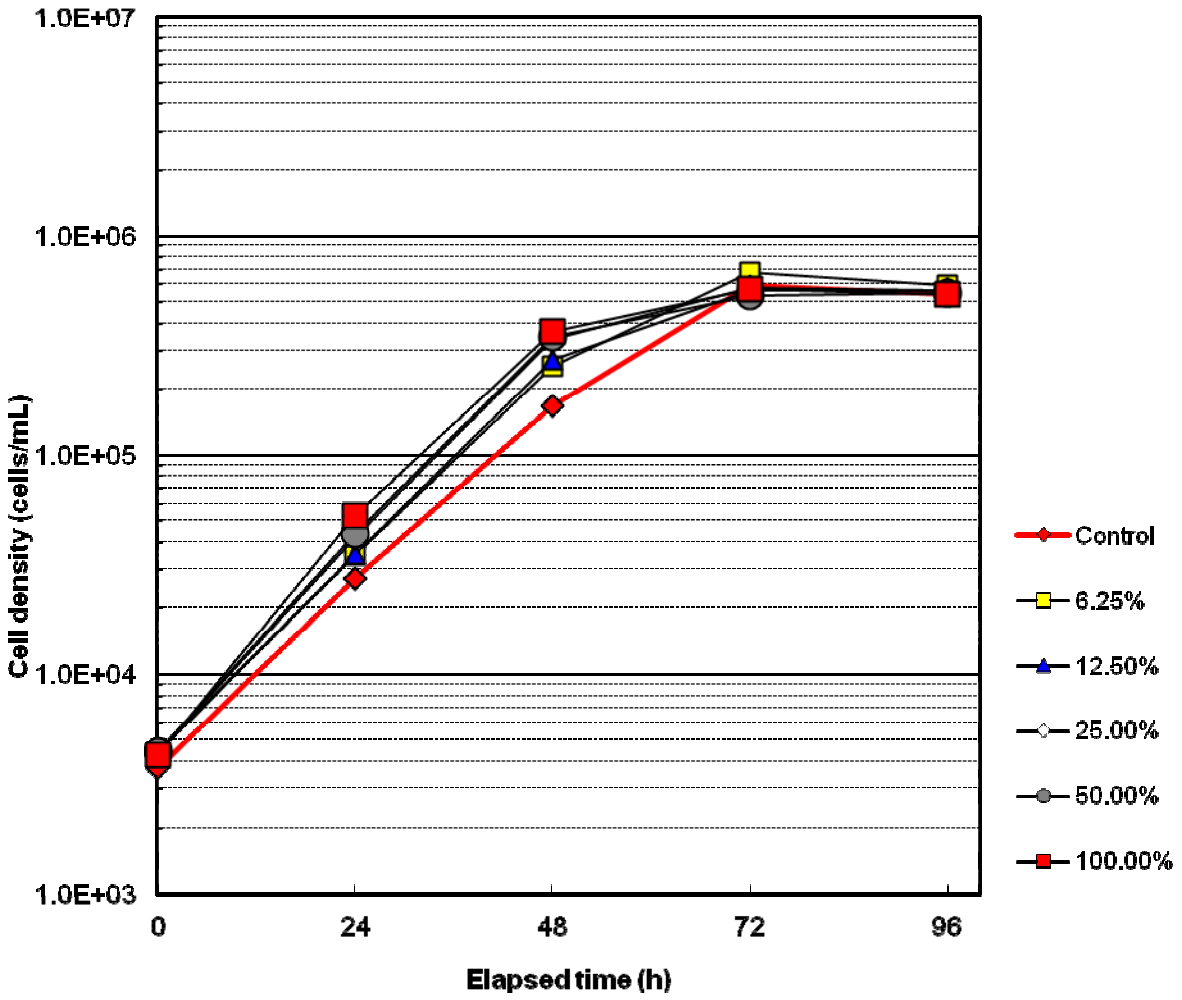

Fig. 4. Growth curves of *Skeletonema costatum* at various concentrations of treated 3-32 psu ballast water. The data of cell density was transformed to log value.

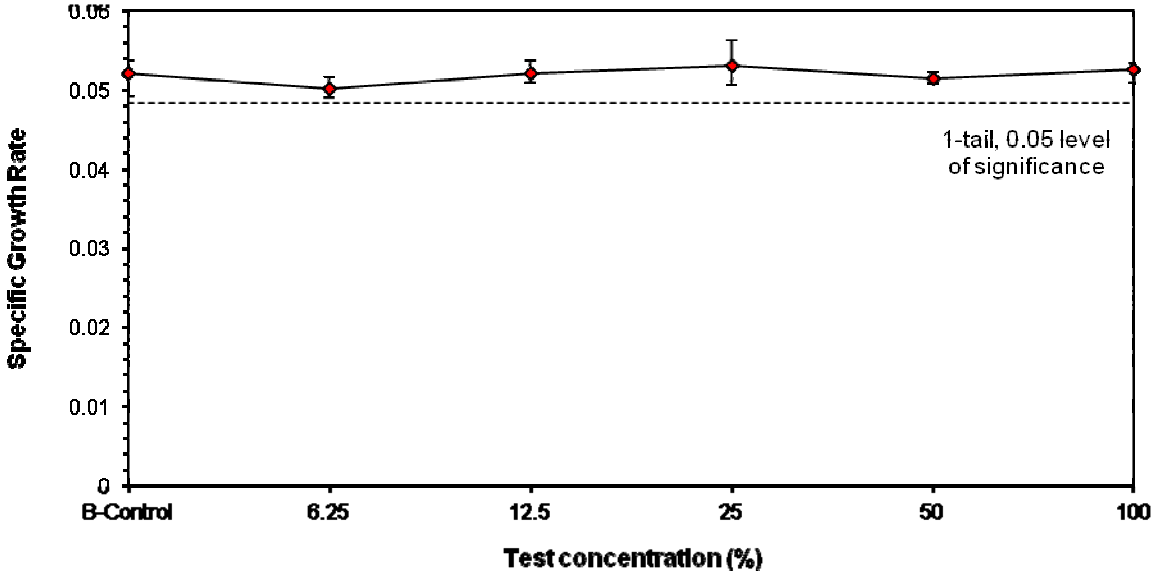

Fig. 5. Plot of the concentration-response data at the end of the experiment with *Skeletonema costatum* in > 32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

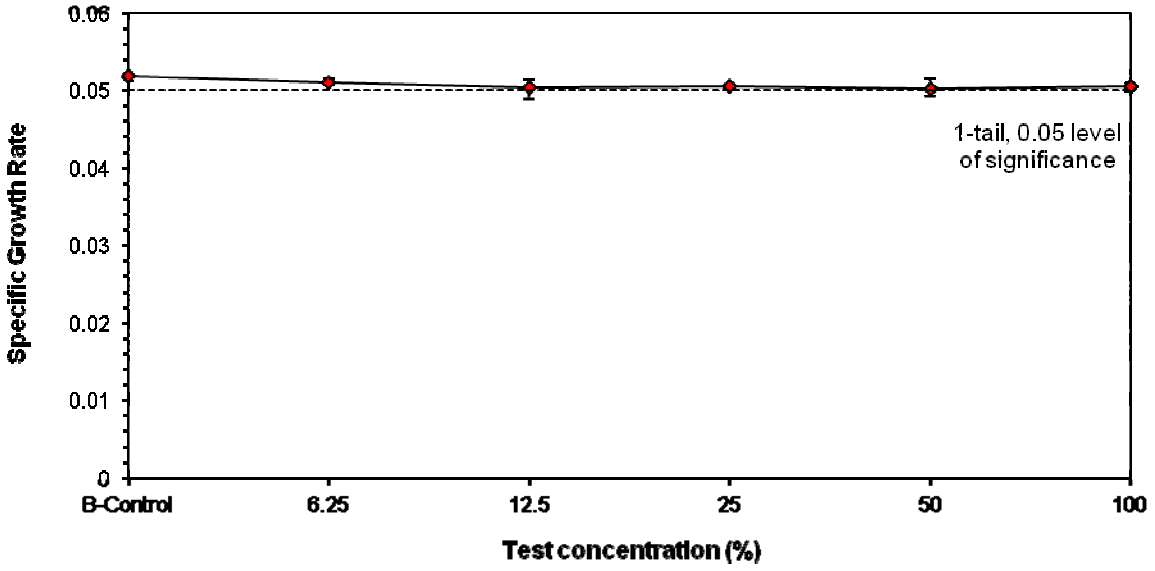

Fig. 6. Plot of the concentration-response data at the end of the experiment with *Skeletonema costatum* in 3-32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

ANNEX 1

Preparation of test substance and procedures of the experiment

Fig. 1. For aquatic toxicity test, collection of the control and discharge treated water from the BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

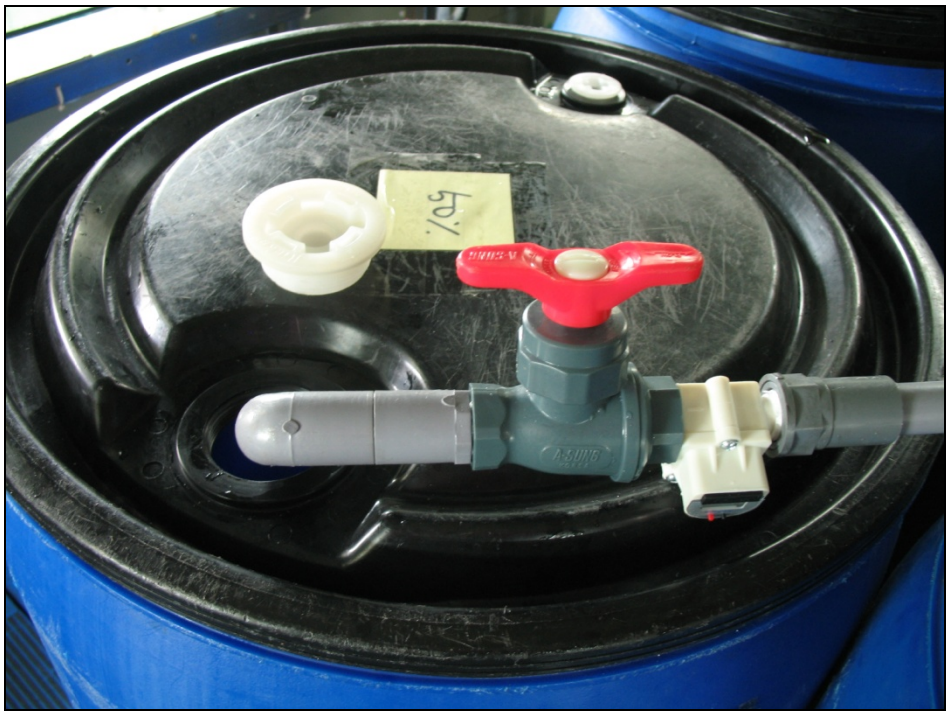

Fig. 3. Preparation of test concentration diluted with non-treated (control) seawater on treated seawater through flow-meter.

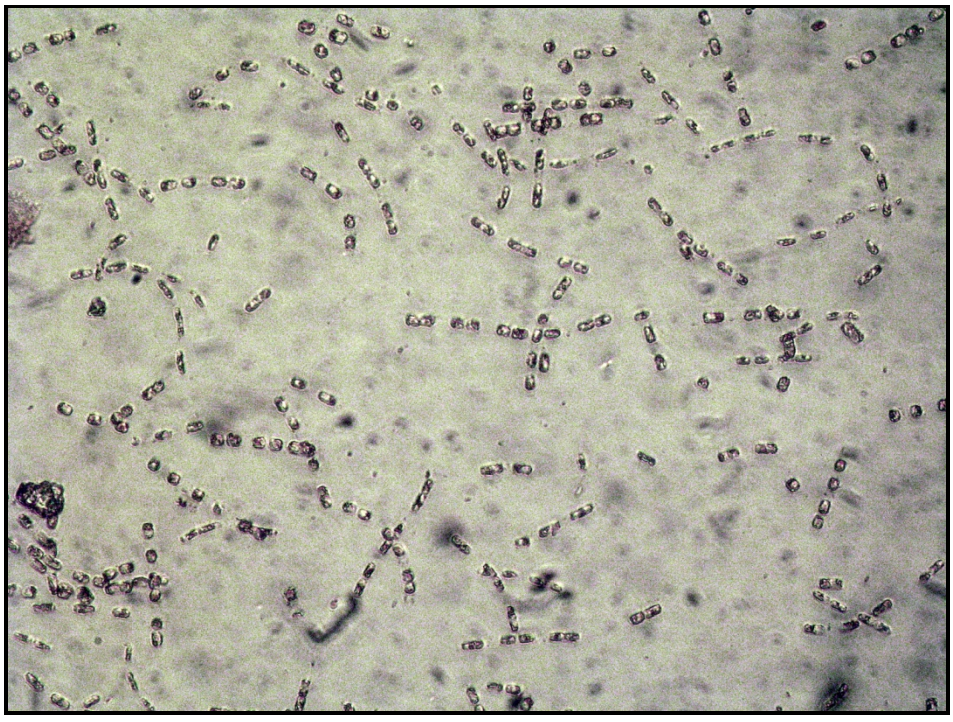

Fig. 4. Test organisms in this study: Aquatic plant (Diatom), *Skeletonema costatum*.

Fig. 5. For the growth inhibition test using the diatom, *Skeletonema costatum*, final preparation of test substance was filtered by 0.45 μm membrane filter.

Fig. 6. The inoculation procedures of *Skeletonema costatum* (① Preparation of test apparatus; ② Sterilizing medium by filter (0.2 μm membrane filter); ③ Inoculation; ④ Incubation at 22 ˚C).

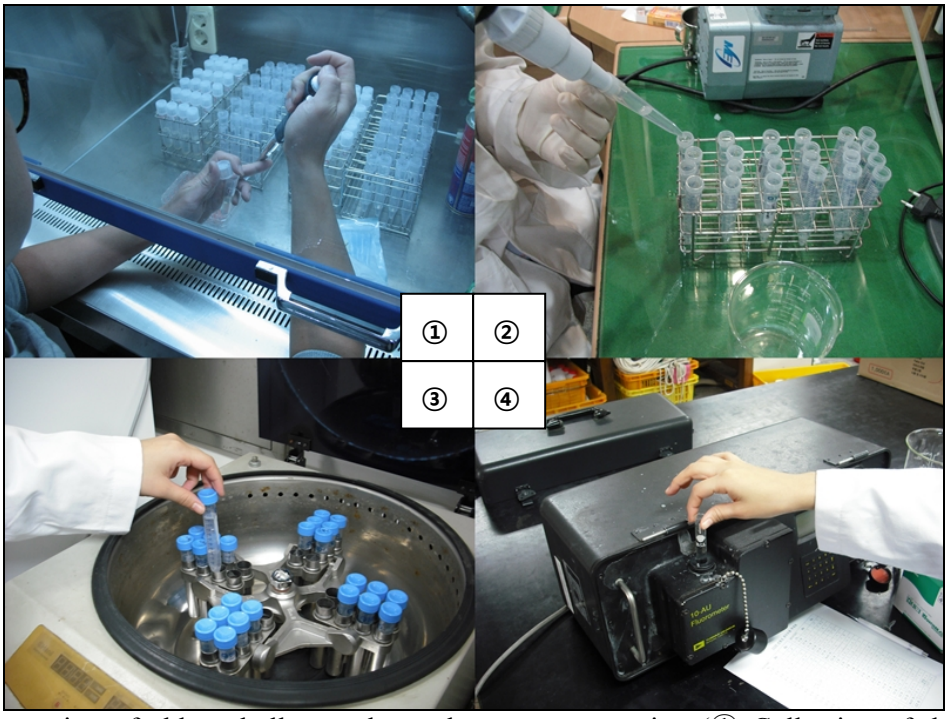

Fig. 7. The extraction of chlorophyll *a* at the each test concentration (① Collecting of the samples; ② Adding the aqueous acetone solution; ③ Centrifuging the extract after storage for a 24 hours in the 4˚C cold and dark room; ④ Estimating the chlorophyll *a*).

2.2 Acute Toxicity Test

2.2.1 ANNEX 1

***Brachionus plicatilis*, Acute Toxicity Test**

Marine Eco-technology Institute Co., Ltd.
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FINAL REPORT

Acute Aquatic Toxicity For > 32 & 3-32 psu discharge treated water of BioViolet™

- Rotifer, *Brachionus plicatilis* Acute Toxicity Test

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GLP STATEMENT AND SIGN

Study title: Acute toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward survival of *Brachionus plicatilis*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

All procedure of this study was carried out following GLP regulation:

- "OECD Principles of Good Laboratory Practice"
  Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

This study was conducted on the basis of 'the Study Plan' complied and the procedure described in 'the Study Plan' for this report was carried out under 'the Responsibility of Study Director'. This report was built on the basis of raw data from the study.

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Study Director Sang-Hee Shin (signature)

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Test Facility Manager Min Ho Son (signature)

GUARANTEE OF ASSURANCE

Study title: Acute toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward survival of *Brachionus plicatilis*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

Quality assurance personnel of Marine Eco-technology Institute Co., Ltd., inspected as follows. Inspection was carried out on the basis of the Standard Operation Procedure and each inspection results were reported to Study Director and Test Facility Manager.

Types of inspection and the date inspection results were reported to the Test Facility Management and the Study Director as follow:

Type of Inspections	Date of Inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011. 02. 28	2011. 02. 28
Test organisms	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Storage of Test substance	2011. 09. 06 / 11. 07	2011. 09. 06 / 11. 07
Preparation of Test substance	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Exposure	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Observation	2011. 09. 08 / 11. 09	2011. 09. 08 / 11. 09
Record of raw data	2011. 09. 08 / 11. 09	2011. 09. 09 / 11. 10
Draft of final report	2011. 12. 23	2011. 12. 23
Final report	2011. 12. 30	2011. 12. 30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Jin Hee Kim (signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

Page

1. SUMMARY

The purpose of this study was to assess the acute toxic effects of the > 32 and 3-32 psu discharge treated water from the BioVioletTM on the survival of the rotifer, *Brachionus plicatilis*.

The test substances were divided into two different salinities, the one was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. 5 individuals of the rotifers, *Brachionus plicatilis* were exposed at the various test concentrations (0.00 % (non-discharge treated water), 6.25, 12.50, 25.00, 50.00 and 100.00 % (discharge treated water)) for 24 hours. Dead rotifer was observed and recorded using a microscopy after 24 hour. The raw data of survival from this experiment was used for calculation of LCx values.

For the >32 psu discharge treated water, the mean survival proportion all test concentrations including the control was 100.0±0.0 percent (Mean ± S.D.). For the 3-32 psu discharge treated water, the mean survival proportion all test concentrations including the control was 98.3±5.8 ~ 100.0±0.0 percent (Mean ± S.D.).

There were not was not significantly different between survival proportion in control and test concentrations. Therefore, for > 32 and 3-32 psu discharge treated water, NOEC, LOEC and 24h-LC50 values at the end of the experiment were equal to or greater than 100.00 % discharge treated water

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have acute toxic effects on the survival of the rotifer, *Brachionus plicatilis*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 11. 07
Separation of neonates for test	2011. 11. 08
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08
Exposure date	2011. 11. 08
Evaluation survival	2011. 11. 09
Experimental completion	2011. 11. 09
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Reference toxicant test	2011. 08. 29 ~ 08. 30
Induced to hatch for cysts	2011. 09. 06
Separation of neonates for test	2011. 09. 07
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation survival	2011. 09. 08
Experimental completion	2011. 09. 08
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For the >32 and 3-32 psu discharge treated water, Kolmogorove D Test and Bartlett's Test were used to determine normality of variance of survival rate and equal variance, Steel's Many-One Rank Test was used to determine the NOEC and LOEC endpoint (survival rate). Differences in all tests were considered to be significant at α=0.05. TOXCALC 5.0 program (Tidepool scientific software, USA) was used program for above statistic analysis.

4. RESULTS

4.1 Reference toxicant test (Table 1)

The value of 24h-LC50 in reference toxin test with rotifer, *Brachionus plicatilis* was 268.4 mg/L as required by the precision object that ranged from 209.0 to 559.9 mg/L.

4.2 Dissolved oxygen, pH and Temperature

4.2.1 Discharge treated water - > 32 psu (Table 2 ~ 4)

For the > 32 psu discharge treated water from the BioViolet^{TM}, DO value was ranged from 6.12 to 6.61 mg/L at the beginning experiment and from 4.56 to 5.37 mg/L at the end of the experiment. pH value was ranged from 7.70 to 7.75 at the beginning experiment and from 7.73 to 7.85 at the end of the experiment. Mean temperature was 25.06 ± 0.28 ˚C (Mean ± S.D.).

4.2.2 Discharge treated water - 3-32 psu (Table 5 ~ 7)

For the 3-32 psu discharge treated water from the BioViolet^{TM}, DO value was ranged from 5.01 to 5.83 mg/L at the beginning experiment and from 5.20 to 6.01 mg/L at the end of the experiment. pH value was ranged from 7.41 to 7.63 at the beginning experiment and from 7.72 to 7.80 at the end of the experiment. Mean temperature was 25.09 ± 0.31 ˚C (Mean ± S.D.).

4.3 Survival proportion

4.3.1 Discharge treated water - > 32 psu (Table 8, 9)

After 24 hours, test organisms survived in all test concentrations including the control. Therefore, the mean survival proportion all test concentrations including the control was 100.0±0.0 (Mean ± S.D.) percent.

4.3.2 Discharge treated water - 3-32 psu (Table 10, 11)

After 24 hours, the only one individual of rotifer died in the control, 25.00 % and 100.0 % test concentration. Therefore, the mean survival proportion all test concentrations including the control was 98.3±5.8 ~ 100.0±0.0 (Mean ± S.D.) percent.

4.4 Plotting of the concentration response curve (Fig. 1,2)

The raw data of proportion of survival from the each test concentration of the >32 and 3-32 psu discharge treated water was plotted on the graph.

4.5 NOEC, LOEC and LCx (Table 12)

There were not significantly different between survival proportion of control and test concentrations ($p>0.05$). Therefore, NOEC, LOEC and 24h-LC50 values at the end of the experiment were equal to or greater than 100.00 % both the > 32 and 3-32 psu discharge treated water.

4.6 Other observation

Any abnormal appearance or behavior of the rotifer, *Brachionus plicatilis* during the experiment period was not observed.

5. DISCUSSION AND CONCLUSION

The survival of rotifer, *Brachionus plicatilis* on the > 32 and 3-32 psu discharge treated water from the BioVioletTM system was assessed.

For the >32 psu discharge treated water, the mean survival proportion all test concentrations including the control was 100.0±0.0 (Mean ± S.D.) percent. For the 3-32 psu discharge treated water, the mean survival proportion all test concentrations including the control was 98.3±5.8 ~ 100.0±0.0 (Mean ± S.D.) percent.

There were not was not significantly different between survival proportion in control and test concentrations. Therefore, for > 32 and 3-32 psu discharge treated water, NOEC, LOEC and 24h-LC50 values at the end of the experiment were equal to or greater than 100.00 % discharge treated water.

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have acute toxic effects on the survival of the rotifer, *Brachionus plicatilis*.

6. REFERENCE

ASTM. 2004. Standard guide for acute toxicity test with the rotifer *Brachionus*. E-1440-91. 8pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

TABLES

Table 1. Reference toxicant test result with rotifer, *Brachionus plicatilis* for precision object

Test substance	24h-LC50 (mg/L)	95 % confidence of limit (mg/L)	
		lower	upper
Potassium dichromate	268.4	241.9	297.4

Table 2. For > 32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	6.15	6.24	6.52	6.61	6.21	6.12
Final	4.97	5.12	4.56	4.97	4.74	5.37

Table 3. For > 32 psu discharge treated water, pH value of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	7.74	7.75	7.74	7.73	7.72	7.70
Final	7.73	7.77	7.78	7.84	7.85	7.83

Table 4. For > 32 psu discharge treated water, temperature (˚C) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	25.30	24.90	24.80	24.90	24.80	24.80
Final	25.60	25.10	25.50	25.10	24.80	25.10

Table 5. For 3-32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	5.83	5.24	5.39	5.30	5.16	5.01
Final	6.01	6.01	5.20	5.60	5.54	5.28

Table 6. For 3-32 psu discharge treated water, pH value of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	7.56	7.41	7.54	7.58	7.61	7.63
Final	7.78	7.73	7.72	7.77	7.80	7.76

Table 7. For 3-32 psu discharge treated water, temperature (˚C) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	24.80	24.90	25.10	25.00	24.80	24.90
Final	25.70	25.70	24.90	25.10	25.20	25.00

Table 8. Data of living and dead rotifer, *Brachionus plicatilis*, exposed to > 32 psu discharge treated water for 24 hours

Concentration	Control		6.25 %		12.50%		25.00%		50.00 %		100.00%	
Elapsed time (h)	24		24		24		24		24		24	
Replication	L	D	L	D	L	D	L	D	L	D	L	D
1	5	0	5	0	5	0	5	0	5	0	5	0
2	5	0	5	0	5	0	5	0	5	0	5	0
3	5	0	5	0	5	0	5	0	5	0	5	0
4	5	0	5	0	5	0	5	0	5	0	5	0
5	5	0	5	0	5	0	5	0	5	0	5	0
6	5	0	5	0	5	0	5	0	5	0	5	0
7	5	0	5	0	5	0	5	0	5	0	5	0
8	5	0	5	0	5	0	5	0	5	0	5	0
9	4	0	5	0	5	0	5	0	5	0	5	0
10	5	0	5	0	5	0	5	0	5	0	5	0
11	5	0	5	0	5	0	5	0	5	0	5	0
12	5	0	5	0	5	0	5	0	5	0	5	0
Mean	5	0	5	0	5	0	5	0	5	0	5	0

*L: Living test organism; D: Dead test organism

Table 9. Survival proportion (%) for the rotifer, *Brachionus plicatilis*, exposed to > 32 psu discharge treated water for 24 hours

Concentration	Control		6.25 %		12.50 %		25.00 %		50.00 %		100.00 %	
Elapsed time(h)	0	24	0	24	0	24	0	24	0	24	0	24
Mean	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
STDEV(±)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

*STDEV: Standard deviation

Table 10. Data of living and dead rotifer, *Brachionus plicatilis*, exposed to 3-32 psu discharge treated water for 24 hours

Concentration	Control		6.25 %		12.50%		25.00%		50.00 %		100.00%	
Elapsed time (h)	24		24		24		24		24		24	
Replication	L	D	L	D	L	D	L	D	L	D	L	D
1	5	0	5	0	5	0	4	1	5	0	5	0
2	5	0	5	0	5	0	5	0	5	0	5	0
3	5	0	5	0	5	0	5	0	5	0	5	0
4	5	0	5	0	5	0	5	0	5	0	4	1
5	5	0	5	0	5	0	5	0	5	0	5	0
6	5	0	5	0	5	0	5	0	5	0	5	0
7	5	0	5	0	5	0	5	0	4	1	5	0
8	5	0	5	0	5	0	5	0	5	0	5	0
9	5	0	5	0	5	0	5	0	5	0	5	0
10	5	0	5	0	5	0	5	0	5	0	5	0
11	5	0	5	0	5	0	5	0	5	0	5	0
12	5	0	5	0	5	0	5	0	5	0	5	0
Mean	5	0	5	0	5	0	5	0	5	0	5	0

*L: Living test organism; D: Dead test organism

Table 11. Survival proportion (%) for the rotifer, *Brachionus plicatilis*, exposed to 3-32 psu discharge treated water for 24 hours

Concentration	**Control**		**6.25 %**		**12.50 %**		**25.00 %**		**50.00 %**		**100.00 %**	
Elapsed time(h)	**0**	**24**	**0**	**24**	**0**	**24**	**0**	**24**	**0**	**24**	**0**	**24**
Mean	100.0	100.0	100.0	100.0	100.0	100.0	100.0	98.3	100.0	100.0	100.0	98.3
STDEV(±)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.8	0.0	0.0	0.0	5.8

*STDEV: Standard deviation

Table 12. NOEC, LOEC and 24h-LC50 values calculated from survival data at the end of the experiment

Test substance	**NOEC (%)**	**LOEC (%)**	**24h-LC50 (%)**	**95 % confidence of limit** (%)	
				lower	**upper**
> 32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-
3-32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-

* -: no data; > 100.0: greater than 100.0 %

FIGURES

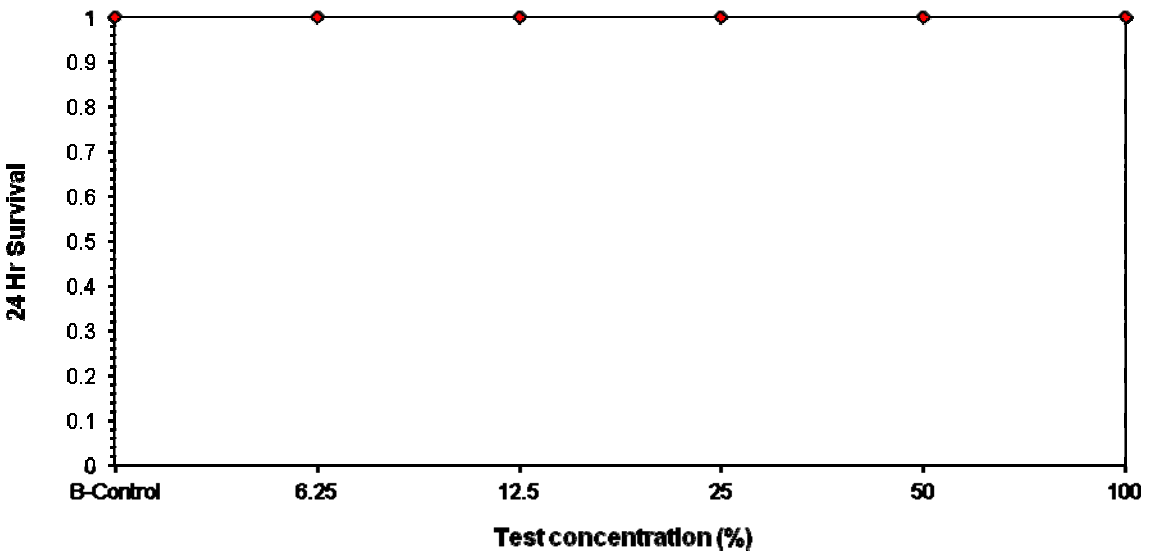

Fig. 1. Plot of the concentration-response (survival proportion) data from the each test concentration for the > 32 psu discharge treated water.

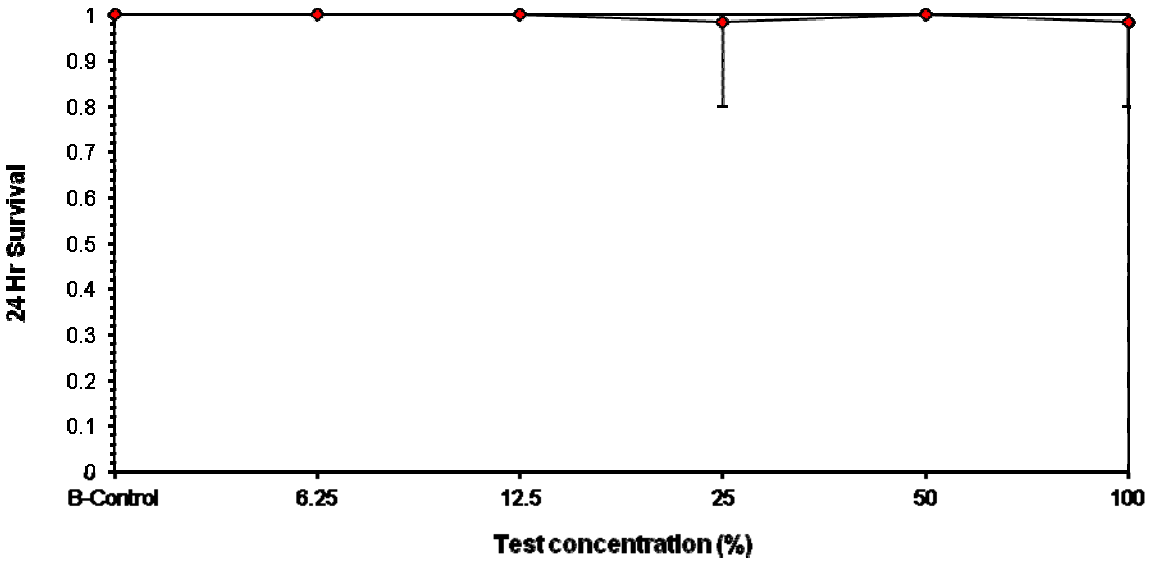

Fig. 2. Plot of the concentration-response (survival proportion) data from the each test concentration for the 3-32 psu discharge treated water.

ANNEX 1

Preparation of test substance and procedures of the experiment

Fig. 1. For aquatic toxicity test, collection of the control (untreated) and treated de-ballasting water from the BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

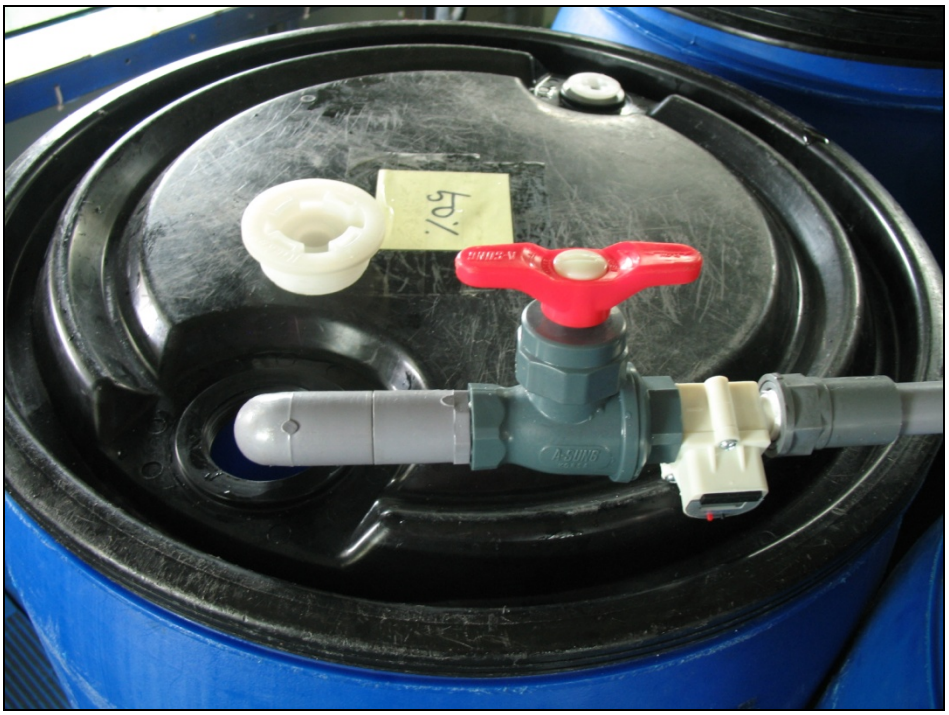

Fig. 3. Preparation of test concentration diluted with non-treated (control) seawater on treated seawater through flow-meter.

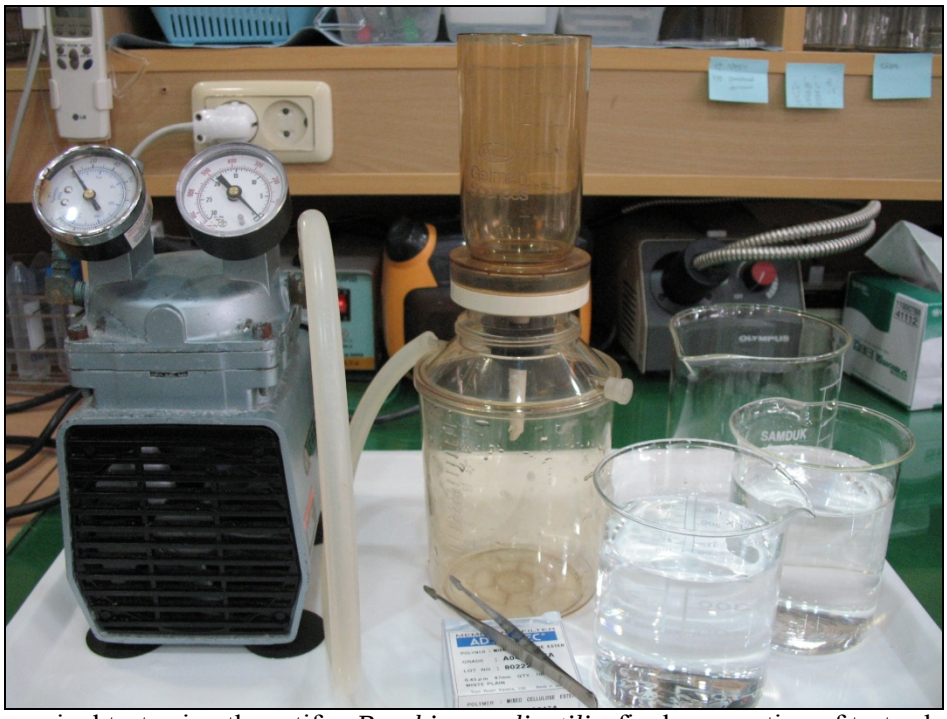

Fig. 4. For the survival test using the rotifer, *Brachionus plicatilis*, final preparation of test substance was filtered by 0.45 μm membrane filter.

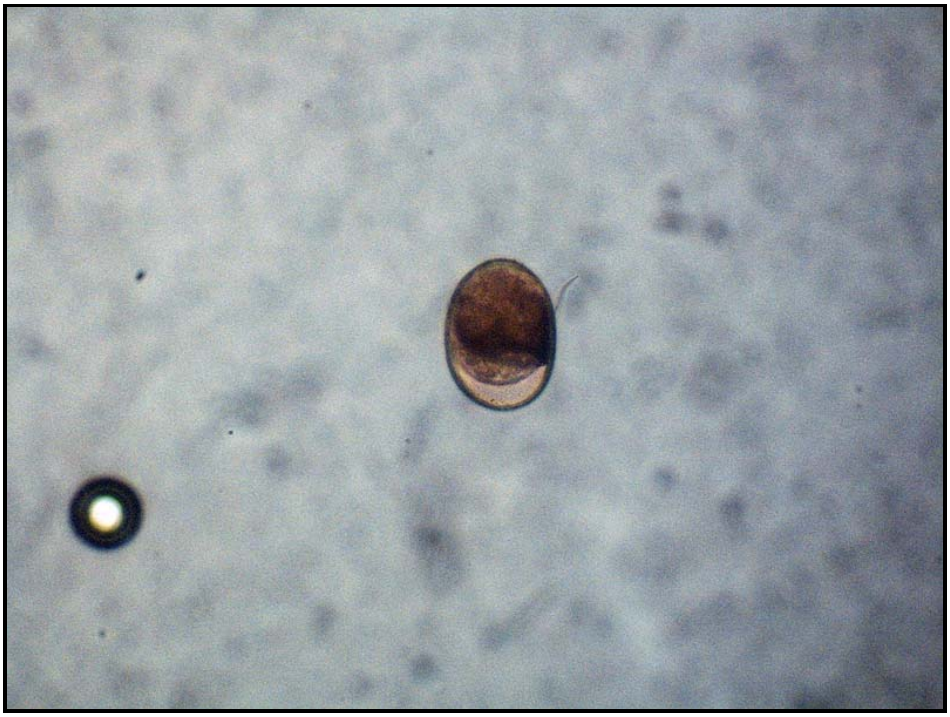

Fig. 5. Test organisms in this study: Cyst and neonates of rotifer, *Brachionus plicatilis*.

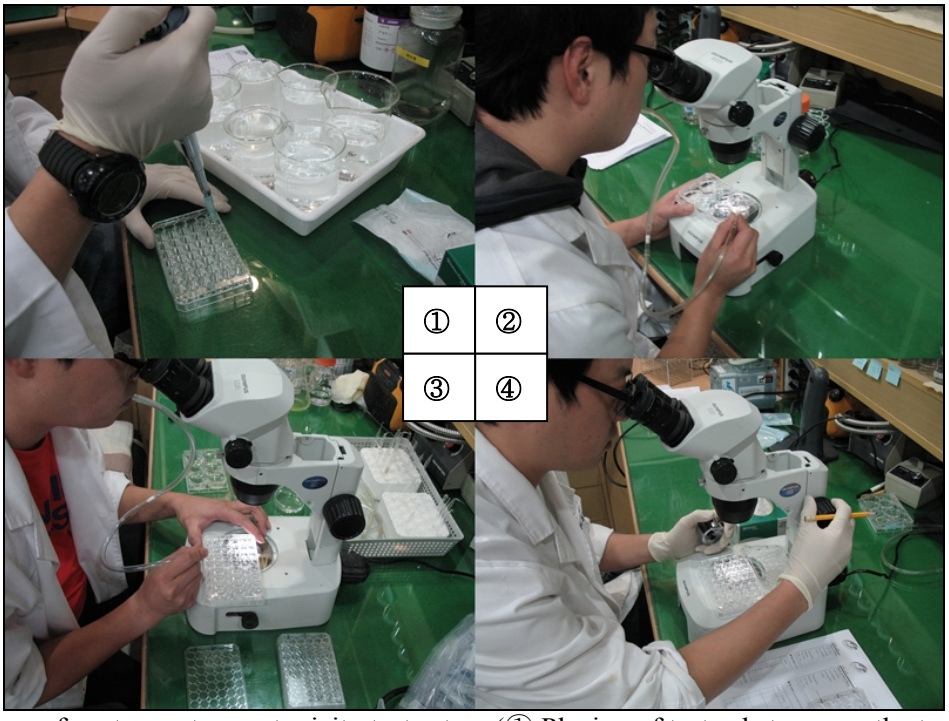

Fig. 6. Procedure of acute crustacean toxicity test set up (① Placing of test substance on the test vessel by pipette; ② Separation of test organisms from hatching chamber; ③ Placing of test organisms on the test vessel by modified pasteur pipette; ④ Observation of the test organisms).

2.2.2 ANNEX 2

The olive flounder fish, *Paralichthys olivaceus*, Acute Toxicity Test

Marine Eco-technology Institute Co., Ltd.
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FINAL REPORT

Acute Aquatic Toxicity For > 32 & 3-32 psu Discharge treated water of BioViolet™

-The olive flounder fish, *Paralichthys olivaceus*, Acute Toxicity Test

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GLP STATEMENT AND SIGN

Study title: Acute toxicity to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward survival of olive flounder fish, *Paralichthys olivaceus*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

All procedure of this study was carried out following GLP regulation:

- "OECD Principles of Good Laboratory Practice"
  Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

This study was conducted on the basis of 'the Study Plan' complied and the procedure described in 'the Study Plan' for this report was carried out under 'the Responsibility of Study Director'. This report was built on the basis of raw data from the study.

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Study Director Jin Hee Kim (signature)

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Test Facility Manager Min Ho Son (signature)

GUARANTEE OF ASSURANCE

Study title: Acute toxicity to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward survival of olive flounder fish, *Paralichthys olivaceus*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

Quality assurance personnel of Marine Eco-technology Institute Co., Ltd., inspected as follows. Inspection was carried out on the basis of the Standard Operation Procedure and each inspection results were reported to Study Director and Test Facility Manager.

Types of inspection and the date inspection results were reported to the Test Facility Management and the Study Director as follow:

Type of Inspections	Date of Inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011 .02.28	2011 .02.28
Test organisms	2011 .08.12 / 10.21	2011 .08.16 / 10.24
Storage of Test substance	2011 .09.06 / 11.07	2011 .09.06 / 11.07
Preparation of Test substance	2011 .09.07 / 11.08	2011 .09.07 / 11.08
Exposure	2011 .09.07 / 11.08	2011 .09.07 / 11.08
Observation	2011 .09.08 / 11.09	2011 .09.08 / 11.09
Record of raw data	2011 .09.08 / 11.09	2011 .09.08 / 11.09
Draft of final report	2011. 12. 23	2011. 12. 23
Final report	2011. 12. 30	2011. 12. 30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Sang-Hee Shin (signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

Page

1. SUMMARY

The purpose of this study was to assess the acute toxic effects of the > 32 and 3-32 psu discharge treated water from the BioVioletTM on the survival of olive flounder, *Paralichthys olivaceus*.

The test substance type was divided into two different salinities, one of them was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. 20 individuals of olive flounder fish, *Paralichthys olivaceus* were exposed at the various test concentration (0.00 % (non-discharge treated water), 6.25, 12.50, 25.0, 50.00 and 100.00 % (discharge treated water)) for 96 hours. The mortality of the olive flounder fish, *P. olivaceus* test organisms was daily observed and recorded. The raw data of living test organisms from this experiment was used for calculation values of LOEC, NOEC and 96h-LC50.

For the > 32 psu discharge treated water, mean cumulative mortality at the end of the experiment was 0.0 percent in the control and all of the test concentrations. For the 3-32 psu discharge treated water, mean cumulative mortality at the end of the experiment was 5.0 percent in the control and those were 18.3, 11.7, 11.7, 1.7 and 0.0 percent in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration.

For > 32 and 3-32 psu discharge treated water, NOEC, LOEC and 96h-LC50 values were equal to or greater than 100.00 % discharge treated water.

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioVioletTM did not have acute toxic effects on the survival of the olive flounder, *Paralichthys olivaceus*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 10. 21
Acclimatization on Lab Condition (> 32 psu)	2011. 10. 21 ~ 11. 05
Settling in the test vessel	2011. 11. 05
Observation of mortality	2011. 11. 05 ~ 11. 07
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08
Exposure date	2011. 11. 08
Evaluation of mortality	2011. 11. 08 ~ 11. 12
Experimental completion	2011. 11. 12
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 08. 12
Acclimatization on Lab Condition (20 psu)	2011. 08. 12 ~ 09. 04
Settling in the test vessel	2011. 09. 04
Observation of mortality	2011. 09. 04 ~ 09. 06
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation of mortality	2011. 09. 07 ~ 09. 11
Experimental completion	2011. 09. 11
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For the > 32 and 3-32 psu discharge treated water, Shapiro-Wilk's Test and Bartlett's Test were used to determine normality and homogeneity of variance of survival rate, respectively. Dunnett's Test was used to determine the NOEC and LOEC endpoint (survival rate). Differences in all tests were considered to be significant at α=0.05. TOXCALC 5.0 program (Tidepool scientific software, USA) was used for above statistic analysis.

4. RESULTS

4.1 Dissolved oxygen, pH, Temperature and Salinity

4.1.1 Discharge treated water - > 32 psu (Table 1~4)

For the > 32 psu discharge treated water from the BioViolet™, DO value was ranged from 5.77 to 6.82 mg/L at the beginning experiment and from 5.12 to 6.74 mg/L at the end of the experiment. pH value was ranged from 7.60 to 7.76 at the beginning experiment and was ranged from 7.52 to 7.84 at the end of the experiment. Mean temperature was 19.823± 0.21 ˚C (mean ± S.D. ˚C) and mean salinity was 34.05 ± 0.22 psu (mean ± S.D. psu).

4.1.2 Discharge treated water - 3-32 psu (Table 5~8)

For the 3-32 psu discharge treated water from the BioViolet™, DO value was ranged from 6.38 to 7.09 mg/L at the beginning experiment and from 6.43 to 6.95 mg/L at the end of the experiment. pH value was ranged from 7.62 to 7.94 at the beginning experiment and was ranged from 7.45 to 7.80 at the end of the experiment. Mean temperature was 19.63 ± 0.34 ˚C (mean ± S.D. ˚C) and mean salinity was 20.25 ± 0.08 psu (mean ± S.D. psu).

4.2 Mortality

4.1.1 Discharge treated water - > 32 psu (Table 9, 10)

Mean cumulative live test organisms at the end of the experiment were 20 individuals in the control and all of the test concentrations. Mean cumulative mortality at the end of the experiment was 0.0 percent in the control and all of the test concentrations.

4.1.2 Discharge treated water - 3-32 psu (Table 11, 12)

Mean cumulative live test organisms at the end of the experiment were 19 individuals in the control and those were 16, 18, 18, 20 and 20 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration. Mean cumulative mortality at the end of the experiment was 5.0 percent in the control and those were 18.3, 11.7, 11.7, 1.7 and 0.0 percent in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration.

4.3 Plotting of the concentration response curve (Fig. 1, 2)

The mortality data on the each test concentration of >32 and 3-32 psu discharge treated water was plotted on the graph.

4.4 NOEC, LOEC and LCx (Table 13)

There were no significantly different between survival rates of control and test concentrations at the end of the experiment ($p>0.05$). Therefore NOEC, LOEC and 96h-LC50 values both the > 32 and 3-32 psu discharge treated water were equal to or greater than 100.00 % in this study.

4.5 Other observation

Any abnormality, such as loss of equilibrium, swimming behavior, respiratory function, pigmentation of the olive flounder fish, *Paralichthys olivaceus* was not observed during the experiment period.

5. DISCUSSION AND CONCLUSION

The mortality on the > 32 and 3-32 psu discharge treated water from the BioViolet^{TM} using olive founder fish, *Paralichthys olivaceus* was assessed for 96 hours.

For the > 32 psu discharge treated water, mean cumulative mortality at the end of the experiment was 0.0 percent in the control and all of the test concentrations. For the 3-32 psu discharge treated water, mean cumulative mortality at the end of the experiment was 5.0 percent in the control and those were 18.3, 11.7, 11.7, 1.7 and 0.0 percent in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration.

For > 32 and 3-32 psu discharge treated water, NOEC, LOEC and 96h-LC50 values based on the survival data were equal to or greater than 100.00% discharge treated water.

Therefore, it was assessed that the > 32 and 3-32 psu discharge treated water from the BioViolet^{TM} did not have acute toxic effects on the survival of the olive flounder, *Paralichthys olivaceus*.

6. REFERENCE

OECD guidelines for the testing of chemicals-203. Fish, Acute Toxicity Test. 9pp.

U.S. EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

TABLES

Table 1. For >32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	6.09	6.54	6.46	**6.36**	6.82	6.48	6.07	**6.46**	6.40	5.81	5.77	**5.99**
24	6.21	6.65	5.59	**6.15**	5.64	5.46	5.57	**5.56**	6.97	6.06	6.54	**6.52**
48	6.10	6.39	6.13	**6.21**	6.82	5.88	5.62	**6.11**	6.40	5.91	5.85	**6.05**
72	5.57	6.68	6.49	**6.25**	6.96	5.83	5.67	**6.15**	6.50	6.04	5.42	**5.99**
96	6.02	5.84	6.54	**6.13**	6.54	6.20	5.12	**5.95**	5.84	5.78	5.64	**5.75**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	6.03	6.37	6.40	**6.27**	6.08	5.89	6.53	**6.17**	6.58	6.27	6.30	**6.38**
24	5.91	5.96	6.53	**6.13**	5.48	6.14	5.66	**5.76**	5.58	5.65	6.56	**5.93**
48	6.19	6.40	6.09	**6.23**	6.23	5.82	6.08	**6.04**	5.89	5.69	6.10	**5.89**
72	6.27	6.15	6.87	**6.43**	5.94	6.24	5.21	**5.80**	5.84	5.74	5.90	**5.83**
96	6.74	6.64	6.42	**6.60**	5.80	6.14	5.43	**5.79**	5.64	5.40	6.41	**5.82**

Table 2. For >32 psu discharge treated water, pH value of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	7.60	7.63	7.67	**7.63**	7.68	7.70	7.72	**7.70**	7.76	7.75	7.70	**7.74**
24	7.63	7.64	7.64	**7.64**	7.68	7.63	7.61	**7.64**	7.69	7.65	7.68	**7.67**
48	7.59	7.62	7.64	**7.62**	7.67	7.61	7.62	**7.63**	7.71	7.68	7.66	**7.68**
72	7.65	7.77	7.82	**7.75**	7.84	7.77	7.73	**7.78**	7.85	7.73	7.77	**7.78**
96	7.52	7.66	7.78	**7.65**	7.80	7.65	7.72	**7.72**	7.84	7.74	7.80	**7.79**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	7.73	7.71	7.70	**7.71**	7.71	7.74	7.70	**7.72**	7.72	7.73	7.75	**7.73**
24	7.68	7.69	7.66	**7.68**	7.62	7.64	7.65	**7.64**	7.61	7.55	7.61	**7.59**
48	7.65	7.61	7.67	**7.64**	7.70	7.61	7.66	**7.66**	7.63	7.72	7.60	**7.65**
72	7.78	7.80	7.79	**7.79**	7.75	7.82	7.78	**7.78**	7.80	7.70	7.76	**7.75**
96	7.82	7.80	7.79	**7.80**	7.84	7.80	7.80	**7.81**	7.81	7.69	7.79	**7.76**

Table 3. For >32 psu discharge treated water, temperature (˚C) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	19.30	19.70	20.10	**19.70**	19.80	20.00	20.20	**20.00**	19.80	20.10	20.10	**20.00**
24	19.60	19.70	19.70	**19.67**	19.80	19.80	19.80	**19.80**	19.60	19.70	19.80	**19.70**
48	19.70	19.70	19.70	**19.70**	19.70	19.80	19.80	**19.77**	19.70	19.70	19.80	**19.73**
72	19.60	19.60	19.60	**19.60**	19.60	19.70	19.80	**19.70**	19.70	19.70	19.70	**19.70**
96	19.50	19.60	19.70	**19.60**	19.60	19.70	19.70	**19.67**	19.70	19.70	19.70	**19.70**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	19.90	19.80	20.00	**19.90**	20.10	20.20	19.90	**20.07**	19.50	19.70	19.20	**19.47**
24	19.70	19.70	20.00	**19.80**	20.00	20.00	20.00	**20.00**	19.80	19.80	19.70	**19.77**
48	19.80	19.80	19.90	**19.83**	20.30	20.30	20.20	**20.27**	20.10	19.90	19.80	**19.93**
72	19.80	19.80	20.20	**19.93**	20.30	20.20	20.10	**20.20**	19.80	19.80	19.70	**19.77**
96	19.80	19.80	20.10	**19.90**	20.10	20.00	20.20	**20.10**	20.00	19.80	19.80	**19.87**

Table 4. For > 32 psu discharge treated water, salinity (psu) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	33.90	32.90	34.00	**33.60**	33.50	34.20	34.20	**33.97**	34.20	34.20	34.20	**34.20**
24	34.10	34.10	34.20	**34.13**	34.20	34.20	34.20	**34.20**	34.20	34.20	34.20	**34.20**
48	33.50	34.10	34.10	**33.90**	34.10	34.00	34.10	**34.07**	34.20	34.10	33.90	**34.07**
72	33.80	34.00	34.00	**33.93**	34.10	34.10	34.10	**34.10**	34.00	34.10	34.10	**34.07**
96	33.70	34.00	34.00	**33.90**	34.00	34.00	34.00	**34.00**	34.20	34.00	34.10	**34.10**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	34.20	34.20	34.00	**34.13**	34.10	34.10	34.20	**34.13**	33.90	33.90	34.30	**34.03**
24	34.20	34.20	34.20	**34.20**	34.20	34.30	34.30	**34.27**	34.30	34.30	34.30	**34.30**
48	34.00	34.10	34.00	**34.03**	34.00	34.10	34.00	**34.03**	34.20	33.40	34.20	**33.93**
72	34.00	34.00	33.90	**33.97**	34.10	34.10	34.10	**34.10**	33.60	34.20	34.20	**34.00**
96	34.00	34.00	33.80	**33.93**	34.00	34.10	34.10	**34.07**	33.40	34.10	34.00	**33.83**

Table 5. For 3-32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	6.38	6.58	6.82	**6.59**	7.01	6.87	6.90	**6.93**	6.87	6.83	6.94	**6.88**
24	6.58	6.33	6.44	**6.45**	6.66	6.87	6.74	**6.76**	7.04	6.75	6.88	**6.89**
48	6.45	6.89	6.47	**6.60**	6.54	6.48	6.80	**6.61**	7.06	6.88	6.79	**6.91**
72	6.66	6.37	6.49	**6.51**	6.60	6.49	6.44	**6.51**	6.89	6.74	6.80	**6.81**
96	6.93	6.43	6.74	**6.70**	6.55	6.57	6.63	**6.58**	6.95	6.84	6.78	**6.86**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	7.03	6.82	6.60	**6.82**	7.04	7.00	7.09	**7.04**	7.03	6.88	6.69	**6.87**
24	6.44	6.65	6.27	**6.45**	6.64	6.70	6.84	**6.73**	7.10	7.08	7.03	**7.07**
48	6.48	7.03	6.92	**6.81**	6.88	6.48	7.00	**6.79**	6.81	6.69	6.79	**6.76**
72	6.55	6.89	6.91	**6.78**	6.84	6.99	6.81	**6.88**	6.98	6.66	6.80	**6.81**
96	6.67	6.74	6.80	**6.74**	6.79	6.51	6.74	**6.68**	6.79	6.70	6.77	**6.75**

Table 6. For 3-32 psu discharge treated water, pH value of 3-32 psu test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	7.62	7.69	7.83	**7.71**	7.79	7.75	7.76	**7.77**	7.79	7.79	7.94	**7.84**
24	7.68	7.70	7.66	**7.68**	7.71	7.71	7.73	**7.72**	7.73	7.73	7.68	**7.71**
48	7.74	7.72	7.72	**7.73**	7.64	7.69	7.66	**7.66**	7.65	7.77	7.70	**7.71**
72	7.65	7.68	7.84	**7.72**	7.71	7.54	7.75	**7.67**	7.71	7.72	7.66	**7.70**
96	7.70	7.72	7.80	**7.74**	7.74	7.71	7.58	**7.68**	7.74	7.45	7.74	**7.64**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	7.76	7.74	7.73	**7.74**	7.80	7.78	7.75	**7.78**	7.74	7.73	7.74	**7.74**
24	7.68	7.58	7.65	**7.64**	7.68	7.58	7.66	**7.64**	7.67	7.68	7.70	**7.68**
48	7.71	7.72	7.77	**7.73**	7.71	7.72	7.71	**7.71**	7.68	7.69	7.70	**7.69**
72	7.72	7.73	7.71	**7.72**	7.73	7.72	7.71	**7.72**	7.69	7.70	7.71	**7.70**
96	7.73	7.72	7.74	**7.73**	7.74	7.73	7.72	**7.73**	7.72	7.71	7.68	**7.70**

Table 7. For 3-32 psu discharge treated water, temperature (˚C) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	19.50	19.40	19.90	**19.60**	20.10	20.40	20.40	**20.30**	20.30	20.30	20.10	**20.23**
24	19.20	19.10	19.10	**19.13**	19.30	19.40	19.40	**19.37**	19.40	19.30	19.20	**19.30**
48	19.50	19.40	19.50	**19.47**	19.60	19.70	19.70	**19.67**	19.70	19.70	19.50	**19.63**
72	19.80	20.00	20.10	**19.97**	19.70	19.80	19.70	**19.73**	19.70	19.90	19.60	**19.73**
96	19.90	20.00	20.00	**19.97**	19.70	19.80	19.80	**19.77**	19.80	20.00	19.60	**19.80**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	20.00	19.90	19.00	**19.63**	20.30	20.10	19.90	**20.10**	19.30	19.20	19.90	**19.47**
24	19.20	19.20	19.40	**19.27**	19.40	19.30	19.20	**19.30**	19.10	19.00	19.00	**19.03**
48	19.40	19.40	19.70	**19.50**	19.70	19.60	19.50	**19.60**	19.30	19.30	19.20	**19.27**
72	19.80	19.50	19.70	**19.67**	19.80	19.60	19.80	**19.73**	19.50	19.60	19.30	**19.47**
96	19.80	19.60	19.80	**19.73**	20.00	20.00	20.10	**20.03**	19.50	19.50	19.40	**19.47**

Table 8. For 3-32 psu discharge treated water, salinity (psu) of test concentration series.

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	20.10	20.10	20.10	**20.10**	20.10	20.20	20.20	**20.17**	20.20	20.20	20.20	**20.20**
24	20.40	20.30	20.30	**20.33**	20.30	20.30	20.30	**20.30**	20.30	20.30	20.30	**20.30**
48	20.30	20.20	20.20	**20.23**	20.30	20.30	20.30	**20.30**	20.30	20.30	20.40	**20.33**
72	20.10	20.20	20.20	**20.17**	20.30	20.30	20.20	**20.27**	20.30	20.20	20.30	**20.27**
96	20.20	20.10	20.20	**20.17**	20.30	20.40	20.20	**20.30**	20.30	20.20	20.30	**20.27**
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	20.20	20.20	20.20	**20.20**	20.20	20.10	20.20	**20.17**	20.30	20.00	20.30	**20.20**
24	20.30	20.30	20.10	**20.23**	20.30	20.20	20.30	**20.27**	20.30	20.30	20.30	**20.30**
48	20.20	20.30	20.30	**20.27**	20.30	20.30	20.30	**20.30**	20.30	20.30	20.30	**20.30**
72	20.20	20.30	20.30	**20.27**	20.30	20.30	20.30	**20.30**	20.30	20.30	20.30	**20.30**
96	20.20	20.20	20.30	**20.23**	20.20	20.30	20.20	**20.23**	20.20	20.20	20.30	**20.23**

Table 9. Live (L) and dead (D) data of *Paralichthys olivaceus*, exposed to > 32 psu discharge treated water during the experiment period

E. T.* (h)	Control								6.25 %								12.50 %							
	1		2		3		Mean		1		2		3		Mean		1		2		3		Mean	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
24	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
48	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
72	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
96	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
E. T. (h)	25.00 %								50.00 %								100.00 %							
	1		2		3		Mean		1		2		3		Mean		1		2		3		Mean	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
24	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
48	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
72	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
96	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0

* E.T. : Elapsed time; L: living test organism; D: dead test organism

Table 10. Mortality of *Paralichthys olivaceus*, exposed to > 32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
96	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Elapsed time (h)	25.00 %				50.00 %				100.00 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
48	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
72	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
96	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Table 11. Live (L) and dead (D) data of *Paralichthys olivaceus*, exposed to 3-32 psu discharge treated water during the experiment period

E. T.* (h)	Control								6.25%								12.50%							
	1		2		3		Mean		1		2		3		Mean		1		2		3		Mean	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
24	20	0	20	0	20	0	20	0	20	0	18	2	20	0	19	1	20	0	20	0	20	0	20	0
48	20	0	19	1	20	0	20	0	18	2	18	2	19	1	18	2	20	0	19	1	20	0	20	0
72	20	0	19	1	19	1	19	1	17	3	18	2	19	1	18	2	19	1	19	1	18	2	19	1
96	20	0	19	1	18	2	19	1	16	4	16	4	17	3	16	4	19	1	17	3	17	3	18	2
E. T. (h)	**25.00%**								**50.00%**								**100.00%**							
	1		2		3		Mean		1		2		3		Mean		1		2		3		Mean	
	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0	20	0
24	18	2	20	0	19	1	19	1	20	0	20	0	19	0	20	0	20	0	20	0	20	0	20	0
48	18	2	20	0	18	2	19	1	20	0	20	0	19	0	20	0	20	0	20	0	20	0	20	0
72	18	2	19	1	18	2	18	2	20	0	20	0	19	0	20	0	20	0	20	0	20	0	20	0
96	18	2	18	2	17	3	18	2	20	0	20	0	19	0	20	0	20	0	20	0	20	0	20	0

* E.T. : Elapsed time; L: living test organism; D: dead test organism

Table 12. Mortality of *Paralichthys olivaceus*, exposed to 3-32 psu discharge treated water during the experiment period

Elapsed time (h)	Control				6.25 %				12.50 %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	0.0	0.0	0.0	0.0	0.0	10.0	0.0	3.3	0.0	0.0	0.0	0.0
48	0.0	5.0	0.0	1.7	10.0	10.0	5.0	8.3	0.0	5.0	0.0	1.7
72	0.0	5.0	5.0	3.3	15.0	10.0	5.0	10.0	5.0	5.0	10.0	6.7
96	0.0	5.0	10.0	5.0	20.0	20.0	15.0	18.3	5.0	15.0	15.0	11.7
Elapsed time (h)	**25.00 %**				**50.00 %**				**100.00 %**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24	10.0	0.0	5.0	5.0	0.0	0.0	5.0	1.7	0.0	0.0	0.0	0.0
48	10.0	0.0	10.0	6.7	0.0	0.0	5.0	1.7	0.0	0.0	0.0	0.0
72	10.0	5.0	10.0	8.3	0.0	0.0	5.0	1.7	0.0	0.0	0.0	0.0
96	10.0	10.0	15.0	11.7	0.0	0.0	5.0	1.7	0.0	0.0	0.0	0.0

Table 13. NOEC, LOEC and 96h-LC50 values calculated from survival data at the end of the experiment

Test substance	NOEC (%)	LOEC (%)	96h-LC50 (%)	95 % confidence of limit (%)	
				lower	upper
> 32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-
3-32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-

* -: no data; > 100.0: greater than 100.0 %

FIGURES

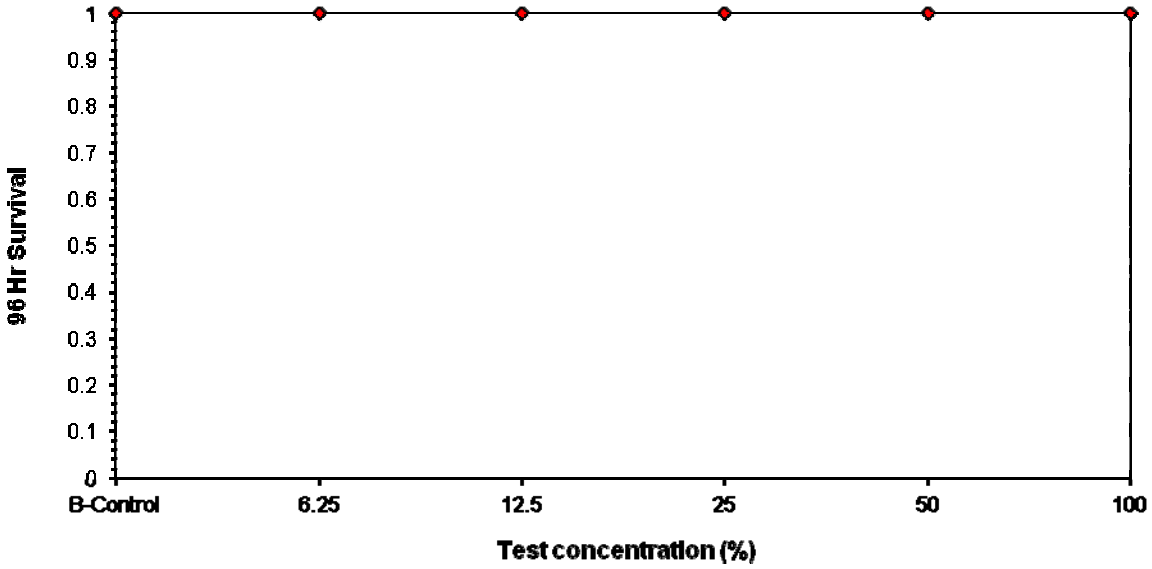

Fig. 1. Plot of the concentration-response data from the each test concentration of the > 32 psu discharge treated water.

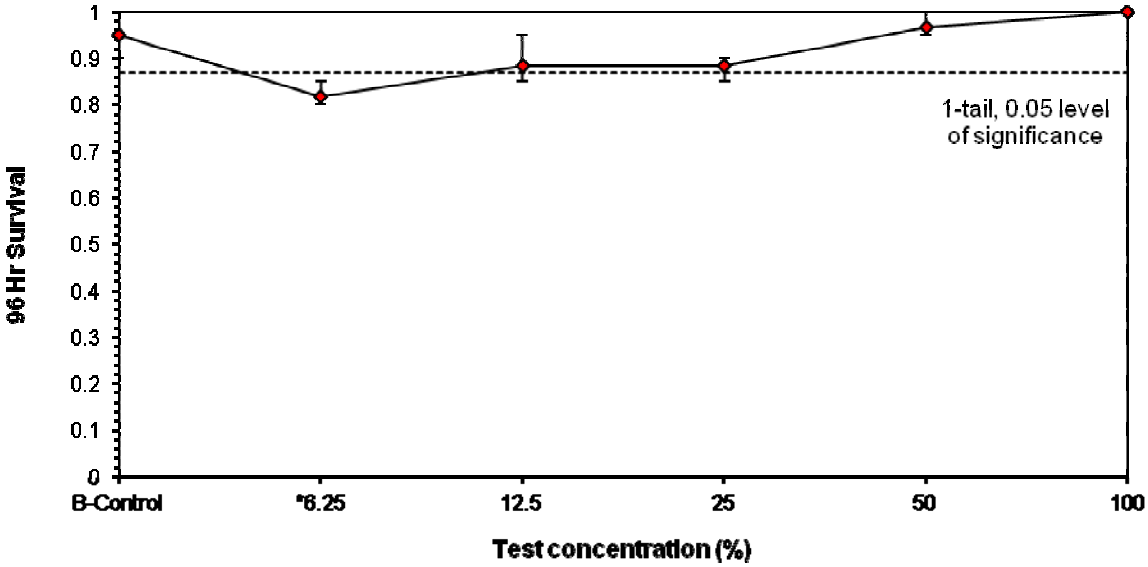

Fig. 2. Plot of the concentration-response data from the each test concentration of the 3-32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

ANNEX 1

Preparation of test substance and procedures of the experiment

Fig. 1. For aquatic toxicity test, collection of the control (untreated) and treated de-ballasting water from the BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

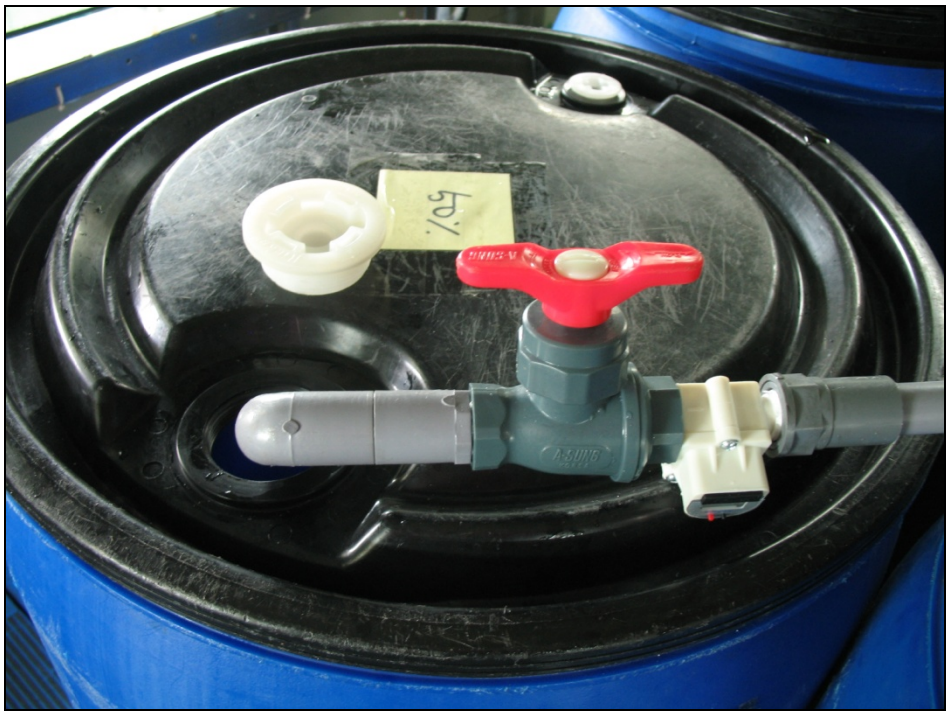

Fig. 3. Preparation of test concentration diluted with non-treated seawater on treated seawater through flow-meter.

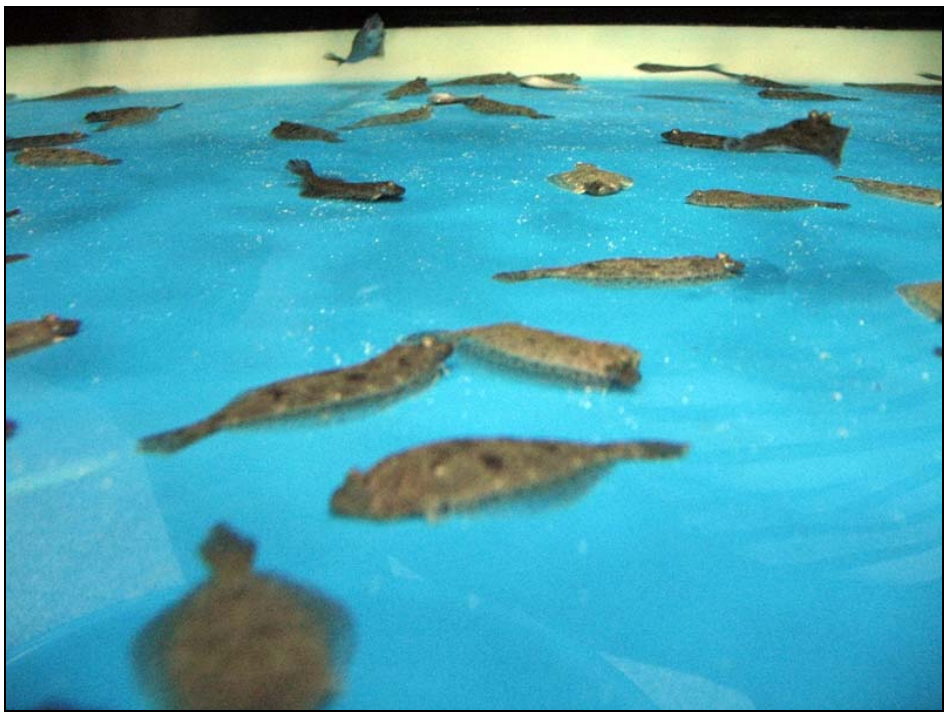

Fig. 4. Test organisms in this study: Olive flounder, *Paralichthys olivaceus*.

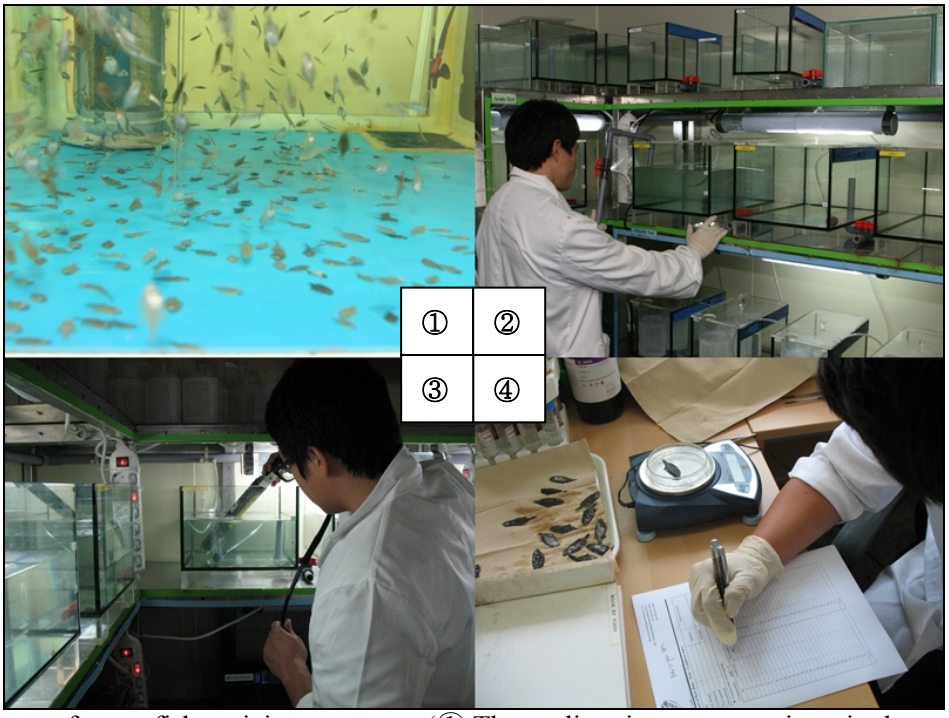

Fig. 5. Procedures of acute fish toxicity test set up (① The acclimating test organisms in the aquarium placed on test facility; ② Test vessels set up; ③ Observation and recording of test organisms and the water quality; ④ Measurement of length and wet weight of the samples).

ANNEX 2

Condition Index

▶ Total length and wet weight of olive flounder juvenile, *Paralichthys olivaceus*, for the > 32 psu discharge treated water from the BioVioletTM

▷ **Concentration :** Control						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.7	0.07	2.9	0.12	2.9	0.15
2	2.8	0.08	2.9	0.11	3.0	0.13
3	3.0	0.10	2.8	0.12	2.9	0.13
4	3.0	0.09	2.9	0.10	3.0	0.11
5	2.5	0.07	2.7	0.10	3.0	0.11
6	2.8	0.10	3.1	0.15	2.9	0.11
7	2.8	0.09	2.4	0.06	3.1	0.12
8	2.7	0.07	3.0	0.13	3.4	0.19
9	2.9	0.12	2.7	0.10	2.9	0.10
10	2.4	0.09	2.7	0.09	3.0	0.12
11	2.5	0.07	2.8	0.13	3.0	0.13
12	2.8	0.09	2.6	0.08	3.0	0.14
13	2.8	0.09	2.5	0.09	3.0	0.13
14	2.7	0.08	2.7	0.07	2.8	0.12
15	2.7	0.08	3.0	0.13	2.8	0.13
16	2.6	0.06	2.5	0.10	3.0	0.16
17	3.0	0.10	2.3	0.07	3.1	0.15
18	2.5	0.06	2.2	0.07	2.8	0.14
19	2.8	0.09	2.4	0.08	3.0	0.12
20	2.7	0.08	2.5	0.09	3.0	0.11
Mean	2.7	0.08	2.7	0.10	3.0	0.13

▷ **Concentration :** 6.25 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.8	0.09	2.5	0.08	2.9	0.13
2	3.0	0.12	2.9	0.10	2.5	0.08
3	2.5	0.08	2.6	0.10	2.6	0.07
4	2.5	0.05	2.7	0.09	2.7	0.10
5	2.4	0.08	2.5	0.10	2.7	0.09
6	2.7	0.08	2.9	0.09	2.3	0.06
7	2.5	0.08	2.5	0.07	2.6	0.12
8	2.6	0.11	2.8	0.11	2.8	0.08
9	2.5	0.08	2.7	0.08	2.3	0.06
10	2.6	0.11	2.4	0.08	2.3	0.06
11	2.4	0.08	3.0	0.10	2.4	0.10
12	2.6	0.07	2.9	0.10	2.3	0.07
13	2.8	0.11	2.5	0.08	2.8	0.06
14	2.8	0.08	2.6	0.08	2.7	0.08
15	2.8	0.08	2.5	0.08	2.7	0.07
16	2.5	0.07	2.6	0.11	2.3	0.06
17	2.6	0.10	2.5	0.08	2.4	0.06
18	2.7	0.08	2.5	0.07	2.4	0.06
19	2.4	0.07	2.7	0.07	2.0	0.01
20	2.7	0.10	2.3	0.06	2.6	0.06
Mean	2.6	0.09	2.6	0.09	2.5	0.07

▷ **Concentration :** 12.50 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.6	0.10	3.0	0.16	2.9	0.14
2	2.7	0.10	2.3	0.09	2.7	0.10
3	2.5	0.10	2.4	0.09	2.7	0.13
4	3.0	0.13	3.1	0.15	2.6	0.11
5	2.8	0.12	2.3	0.10	2.6	0.09
6	3.0	0.16	3.0	0.12	2.8	0.09
7	2.7	0.10	2.6	0.10	2.7	0.10
8	2.6	0.08	2.7	0.12	2.7	0.13
9	2.6	0.09	2.5	0.13	2.4	0.07
10	2.5	0.09	2.6	0.10	2.3	0.08
11	2.5	0.08	2.5	0.08	2.4	0.08
12	2.7	0.09	2.8	0.10	2.3	0.08
13	2.8	0.13	2.5	0.08	2.8	0.13
14	2.6	0.09	3.0	0.11	2.7	0.09
15	2.4	0.09	2.6	0.11	2.5	0.10
16	2.3	0.08	2.8	0.10	2.2	0.06
17	2.6	0.08	2.8	0.11	2.5	0.11
18	2.4	0.09	2.7	0.08	2.0	0.07
19	2.7	0.13	2.8	0.09	2.6	0.12
20	2.6	0.12	2.7	0.07	2.7	0.10
Mean	2.6	0.10	2.7	0.10	2.6	0.10

▷ **Concentration :** 25.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.5	0.10	2.5	0.10	2.5	0.08
2	2.3	0.11	3.2	0.17	2.4	0.09
3	2.6	0.12	2.5	0.09	2.4	0.07
4	2.7	0.11	2.9	0.14	2.3	0.06
5	2.8	0.14	2.6	0.10	2.5	0.07
6	2.8	0.15	2.7	0.12	2.5	0.07
7	2.7	0.14	3.0	0.11	2.4	0.08
8	2.5	0.09	2.8	0.11	2.4	0.07
9	2.6	0.11	2.6	0.10	2.6	0.09
10	2.5	0.08	2.5	0.09	2.3	0.07
11	2.8	0.13	2.9	0.13	2.4	0.08
12	2.5	0.09	2.8	0.13	2.3	0.07
13	2.4	0.09	2.7	0.15	2.4	0.09
14	2.5	0.12	2.6	0.10	2.2	0.06
15	2.5	0.09	2.9	0.13	2.5	0.10
16	2.5	0.14	2.5	0.09	2.5	0.08
17	2.8	0.12	2.9	0.13	2.3	0.06
18	2.3	0.09	2.7	0.12	2.2	0.06
19	2.4	0.09	2.4	0.09	2.5	0.07
20	2.8	0.14	2.6	0.12	2.5	0.08
Mean	2.6	0.11	2.7	0.12	2.4	0.08

▷ **Concentration :** 50.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.4	0.09	2.4	0.08	2.5	0.07
2	2.5	0.08	2.6	0.08	2.6	0.14
3	2.4	0.09	2.7	0.11	2.5	0.09
4	2.4	0.06	2.9	0.13	2.7	0.09
5	2.5	0.08	2.5	0.08	2.8	0.14
6	2.4	0.09	2.6	0.09	2.7	0.13
7	2.5	0.08	2.4	0.08	3.0	0.17
8	2.5	0.08	2.5	0.10	2.5	0.10
9	2.4	0.09	3.0	0.16	3.0	0.16
10	2.5	0.06	2.9	0.10	3.0	0.14
11	2.5	0.08	2.3	0.07	2.8	0.11
12	2.6	0.07	2.9	0.12	3.1	0.15
13	2.2	0.06	2.7	0.08	2.6	0.12
14	2.3	0.07	2.5	0.07	2.6	0.10
15	2.5	0.12	2.5	0.11	2.7	0.12
16	2.2	0.06	2.5	0.10	2.7	0.12
17	2.4	0.08	2.7	0.11	2.6	0.11
18	2.6	0.10	2.7	0.09	2.3	0.07
19	2.4	0.07	2.6	0.08	2.5	0.10
20	2.7	0.10	2.4	0.07	2.8	0.12
Mean	2.4	0.08	2.6	0.10	2.7	0.12

▷ **Concentration :** 100.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	2.3	0.07	2.5	0.09	2.6	0.10
2	2.3	0.08	2.7	0.10	2.4	0.06
3	2.6	0.13	2.5	0.09	2.4	0.09
4	2.3	0.08	2.3	0.05	2.4	0.07
5	2.8	0.15	2.8	0.14	2.5	0.06
6	2.4	0.08	2.7	0.12	2.1	0.06
7	2.1	0.05	2.6	0.11	2.4	0.07
8	2.7	0.12	2.5	0.09	2.5	0.08
9	2.2	0.09	2.6	0.11	2.3	0.08
10	2.3	0.07	2.6	0.09	2.3	0.07
11	2.5	0.11	2.4	0.07	2.7	0.08
12	2.4	0.07	2.5	0.09	2.5	0.09
13	2.2	0.07	2.6	0.12	2.2	0.06
14	2.7	0.12	2.5	0.11	2.2	0.06
15	2.3	0.09	2.6	0.12	2.6	0.09
16	2.4	0.07	2.7	0.12	2.4	0.07
17	2.3	0.09	2.8	0.12	2.6	0.09
18	2.3	0.08	2.6	0.11	2.2	0.06
19	2.7	0.09	2.7	0.12	2.3	0.06
20	2.4	0.06	2.5	0.12	2.6	0.10
Mean	2.4	0.09	2.6	0.10	2.4	0.08

▶ Total length and wet weight of juvenile olive flounder, *Paralichthys olivaceus*, for the 3-32 psu discharge treated water from the BioViolet™

▷ **Concentration :** Control						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	4.3	0.43	4.1	0.30	4.1	0.35
2	4.2	0.41	4.2	0.40	3.7	0.27
3	4.1	0.35	3.8	0.33	4.1	0.34
4	4.5	0.54	4.1	0.38	3.5	0.24
5	4.4	0.48	3.7	0.30	3.2	0.30
6	4.1	0.38	4.0	0.31	3.6	0.32
7	4.6	0.54	4.0	0.35	4.3	0.45
8	4.4	0.48	4.2	0.34	4.4	0.35
9	4.9	0.59	5.0	0.40	4.2	0.43
10	5.2	0.60	4.7	0.39	4.6	0.41
11	5.6	0.74	4.4	0.34	4.8	0.51
12	5.1	0.55	4.3	0.35	4.2	0.41
13	4.0	0.42	4.6	0.36	4.0	0.39
14	4.8	0.45	5.0	0.41	4.1	0.38
15	4.7	0.48	5.1	0.38	4.1	0.35
16	4.4	0.43	4.2	0.33	5.0	0.45
17	4.4	0.45	4.1	0.34	5.1	0.42
18	4.6	0.47	4.6	0.36	4.6	0.50
19	5.0	0.50	5.0	0.42	4.8	0.51
20	4.1	0.39	5.2	0.41	4.7	0.49
Mean	4.6	0.48	4.4	0.36	4.3	0.39

▷ **Concentration :** 6.25 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	3.9	0.34	4.0	0.39	3.9	0.31
2	4.3	0.40	4.0	0.37	3.9	0.34
3	3.8	0.30	4.8	0.49	4.0	0.35
4	3.3	0.31	4.2	0.42	3.7	0.29
5	3.9	0.34	4.5	0.48	4.0	0.38
6	4.0	0.36	4.4	0.50	3.8	0.34
7	4.0	0.39	3.8	0.32	3.9	0.35
8	4.5	0.40	3.9	0.40	3.8	0.33
9	5.0	0.42	4.0	0.43	4.0	0.39
10	5.1	0.46	4.4	0.40	4.5	0.40
11	4.7	0.44	4.6	0.46	4.6	0.44
12	4.3	0.41	4.7	0.39	4.1	0.41
13	4.8	0.40	4.8	0.42	4.3	0.40
14	3.9	0.33	5.0	0.45	4.4	0.41
15	5.0	0.56	5.3	0.50	4.7	0.45
16	5.1	0.50	5.0	0.44	3.8	0.35
17	3.8	0.32	3.9	0.39	5.0	0.49
18	4.3	0.39	4.8	0.41	5.2	0.48
19	4.6	0.42	4.2	0.44	5.7	0.52
20	4.2	0.40	4.6	0.46	3.9	0.37
Mean	4.3	0.39	4.4	0.43	4.3	0.39

▷ **Concentration :** 12.50 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	3.1	0.30	4.0	0.38	4.0	0.37
2	3.2	0.31	4.4	0.40	4.0	0.36
3	3.6	0.33	4.3	0.44	4.2	0.41
4	3.2	0.29	4.0	0.42	4.1	0.38
5	3.4	0.34	4.8	0.41	4.8	0.43
6	3.2	0.38	4.6	0.39	4.7	0.45
7	3.8	0.36	3.9	0.38	4.3	0.46
8	4.0	0.39	4.0	0.42	4.9	0.48
9	4.1	0.40	5.0	0.44	5.0	0.47
10	4.6	0.42	5.1	0.43	4.7	0.48
11	5.0	0.48	5.2	0.42	4.2	0.42
12	4.3	0.41	4.9	0.40	4.9	0.43
13	5.2	0.47	4.2	0.43	4.5	0.39
14	5.4	0.45	4.0	0.41	4.9	0.43
15	3.9	0.39	4.3	0.43	5.0	0.49
16	4.6	0.43	4.7	0.46	3.6	0.36
17	4.8	0.44	5.0	0.46	3.8	0.34
18	4.1	0.41	3.8	0.38	4.8	0.42
19	4.2	0.42	3.4	0.30	5.1	0.50
20	4.6	0.45	3.8	0.37	4.2	0.41
Mean	4.1	0.39	4.4	0.41	4.5	0.42

▷ **Concentration :** 25.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	4.3	0.40	4.9	0.48	3.9	0.34
2	4.0	0.35	4.8	0.47	3.0	0.35
3	3.5	0.28	4.3	0.43	4.4	0.42
4	4.0	0.33	4.2	0.44	3.5	0.35
5	4.1	0.42	4.6	0.46	4.0	0.43
6	4.2	0.39	4.5	0.40	4.2	0.42
7	4.6	0.40	4.0	0.42	4.3	0.39
8	4.4	0.42	3.9	0.39	4.4	0.40
9	3.9	0.40	5.0	0.53	4.6	0.44
10	4.8	0.43	4.6	0.44	4.0	0.39
11	5.3	0.48	3.8	0.39	4.8	0.50
12	4.7	0.42	3.7	0.40	5.2	0.48
13	3.9	0.37	4.8	0.47	5.1	0.53
14	4.4	0.40	5.0	0.44	4.9	0.49
15	3.8	0.37	3.7	0.39	4.7	0.47
16	3.1	0.29	4.0	0.41	3.9	0.39
17	4.9	0.44	3.6	0.33	3.8	0.42
18	4.5	0.40	5.3	0.50	4.0	0.44
19	4.7	0.44	5.0	0.49	4.2	0.43
20	5.0	0.45	3.8	0.41	4.0	0.40
Mean	4.3	0.39	4.4	0.43	4.2	0.42

▷ **Concentration :** 50.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	4.0	0.45	3.7	0.41	4.2	0.42
2	4.1	0.42	4.0	0.41	4.1	0.36
3	4.2	0.41	4.3	0.44	4.0	0.39
4	4.1	0.39	4.0	0.32	3.7	0.33
5	3.6	0.33	3.9	0.31	3.8	0.31
6	4.3	0.41	4.0	0.32	3.8	0.38
7	3.9	0.32	3.6	0.30	3.6	0.30
8	4.4	0.43	4.0	0.34	3.9	0.33
9	4.2	0.42	4.4	0.39	4.3	0.50
10	4.2	0.40	4.5	0.44	4.4	0.42
11	4.3	0.44	5.0	0.48	4.5	0.44
12	4.2	0.43	5.1	0.47	4.8	0.45
13	4.3	0.46	4.7	0.43	5.0	0.50
14	4.9	0.47	3.8	0.38	5.1	0.47
15	5.0	0.43	4.0	0.40	3.9	0.38
16	4.6	0.47	4.3	0.39	4.6	0.44
17	3.9	0.33	4.8	0.48	4.7	0.48
18	3.8	0.39	5.1	0.47	4.8	0.47
19	5.1	0.49	3.9	0.38	4.1	0.39
20	5.0	0.47	4.7	0.40	4.4	0.42
Mean	4.3	0.42	4.3	0.40	4.3	0.41

▷ **Concentration :** 100.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**	**Length (cm)**	**Wet weight (g)**
1	3.9	0.32	4.1	0.42	4.6	0.44
2	4.5	0.44	4.5	0.50	4.0	0.39
3	4.0	0.39	4.3	0.51	4.3	0.40
4	4.5	0.47	4.4	0.57	4.4	0.43
5	4.5	0.42	4.6	0.46	4.5	0.41
6	3.6	0.33	3.9	0.32	4.5	0.43
7	4.2	0.44	3.9	0.35	4.0	0.38
8	4.7	0.48	4.0	0.34	4.3	0.39
9	4.4	0.41	4.0	0.37	4.0	0.37
10	4.5	0.42	4.1	0.39	4.5	0.42
11	4.7	0.50	4.6	0.44	4.3	0.43
12	4.0	0.39	4.8	0.43	4.9	0.45
13	5.0	0.49	5.0	0.51	5.0	0.40
14	5.3	0.51	3.9	0.34	5.1	0.49
15	3.9	0.38	4.8	0.50	3.9	0.33
16	5.0	0.51	4.6	0.44	4.4	0.41
17	4.3	0.44	4.2	0.40	3.8	0.38
18	5.0	0.46	5.0	0.44	4.5	0.42
19	4.8	0.41	5.1	0.51	4.2	0.40
20	4.7	0.43	4.3	0.40	5.0	0.47
Mean	4.5	0.43	4.4	0.43	4.4	0.41

2.3 Chronic Toxicity Test

2.3.1 ANNEX 1

***Brachionus plicatilis*, Chronic Toxicity Test**

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FINAL REPORT

Chronic Aquatic Toxicity For > 32 & 3-32 psu discharge treated water of BioViolet™

- Rotifer, *Brachionus plicatilis* Chronic Toxicity Test

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GLP STATEMENT AND SIGN

Study title: Chronic toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward population growth rate of *Brachionus plicatilis*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

All procedure of this study was carried out following GLP regulation:

- "OECD Principles of Good Laboratory Practice"
  Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

This study was conducted on the basis of 'the Study Plan' complied and the procedure described in 'the Study Plan' for this report was carried out under 'the Responsibility of Study Director'. This report was built on the basis of raw data from the study.

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Study Director Sang-Hee Shin (signature)

2011. 12. 30.

Marine Eco-technology Institute Co., Ltd.
Test Facility Manager Min Ho Son (signature)

GUARANTEE OF ASSURANCE

Study title: Chronic toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioVioletTM toward population growth rate of *Brachionus plicatilis*

Study name: BioVioletTM Study No: BW-DBWT1107-KS

Quality assurance personnel of Marine Eco-technology Institute Co., Ltd., inspected as follows. Inspection was carried out on the basis of the Standard Operation Procedure and each inspection results were reported to Study Director and Test Facility Manager.

Types of inspection and the date inspection results were reported to the Test Facility Management and the Study Director as follow:

Type of Inspections	Date of Inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011. 02. 28	2011. 02. 28
Test organisms	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Storage of Test substance	2011. 09. 06 / 11. 07	2011. 09. 07 / 11. 08
Preparation of Test substance	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Exposure	2011. 09. 07 / 11. 08	2011. 09. 07 / 11. 08
Observation	2011. 09. 08 / 11. 09	2011. 09. 08 / 11. 09
Record of raw data	2011. 09. 08 / 10. 09	2011. 09. 08 / 11. 09
Draft of final report	2011. 12. 23	2011. 12. 23
Final report	2011. 12. 30	2011. 12. 30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Jin Hee Kim (Signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

Page

1. SUMMARY

The purpose of this study was to assess the chronic toxic effects of the > 32 and 3-32 psu discharge treated water from the BioVioletTM on the population growth rate of rotifer, *Brachionus plicatilis*.

The test substances were divided into two different salinities, the one was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. 5 individuals of the rotifers, *Brachionus plicatilis* were exposed at the various test concentrations (0.00 % (non-discharge treated water), 6.25, 12.50, 25.00, 50.00 and 100.00 % (discharge treated water)) for 96 hours. The rotifer was observed daily using microscopy. Dead rotifers were removed after record. The raw data of population growth from this experiment was used for calculation of NOEC, LOEC and ECx values.

For the > 32 psu discharge treated water, the population growth rate of the test organisms in the control was 0.59 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.57, 0.58, 0.58, 0.58 and 0.59, respectively.

For the 3-32 psu discharge treated water, the population growth rate of the test organisms in the control was 0.63 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.64, 0.62, 0.63, 0.62 and 0.61, respectively.

For the >32 psu and 3-32 psu discharge treated water, values of NOEC, LOEC and 96h-EC50 based on population growth rate at the end of experiment were equal to or higher than 100.00 % discharge treated water (Table 13).

Therefore, it was assessed that the > 32 psu and 3-32 psu discharge treated water from the BioVioletTM did not have chronic toxic effects on the population growth of the rotifer, *B. plicatilis*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 11. 07
Separation of neonates for test	2011. 11. 08
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08
Exposure date	2011. 11. 08
Evaluation population growth rate	2011. 11. 08 ~ 11. 12
Experimental completion	2011. 11. 12
Draft of final report	2011. 11. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 09. 06
Separation of neonates for test	2011. 09. 07
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation population growth rate	2011. 09. 07 ~ 09.11
Experimental completion	2011. 09. 11
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For the > 32 and 3-32 psu discharge treated water, Kolmogorov D Test and Bartlett's Test were used to determine normality and homogeneity of variance of survival rate, respectively. Dunnett's Test was used to determine the NOEC and LOEC endpoint (population growth rate). Differences were considered to be significant at α=0.05.

TOXCALC 5.0 program (Tidepool scientific software, USA) was used for above statistic analysis.

4. RESULTS

4.1 Dissolved oxygen, pH and Temperature

4.1.1 Discharge treated water - > 32 psu (Table 1 ~ 3)

For the > 32 psu discharge treated water from the BioVioletTM, DO value was ranged from 6.12 to 6.61 mg/L at the beginning of the experiment and from 4.88 to 5.67 mg/L at the end of the experiment. pH value was ranged from 7.70 to 7.75 at the beginning of the experiment and from 8.02 to 8.09 at the end of the experiment. And mean temperature was 25.08 ± 0.25 ˚C (Mean ± S.D.).

4.1.2 Discharge treated water - 3-32 psu (Table 4 ~ 6)

For the 3-32 psu discharge treated water from the BioVioletTM, DO value was ranged from 5.06 to 5.82 mg/L at the beginning of the experiment and from 5.02 to 5.97 mg/L at the end of the experiment. pH value was ranged from 7.72 to 7.84 at the beginning of the experiment and from 7.72 to 8.07 at the end of the experiment. And mean temperature was 25.18 ± 0.28 ˚C (Mean ± S.D.).

4.2 Population growth rate

4.2.1 Discharge treated water - > 32 psu (Table 7, 8)

The mean number of the rotifer, *Brachionus plicatilis* exposed the > 32 psu discharge treated water for 96 hours in the control was 52 individuals and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 48, 51, 52, 52 and 53 individuals, respectively. The population growth rate of the test organisms in the control was 0.59 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.57, 0.58, 0.58, 0.58 and 0.59, respectively.

4.2.2 Discharge treated water - 3-32 psu (Table 10, 11)

The mean number of the rotifer, *Brachionus plicatilis* exposed the 3-32 psu discharge treated water for 96 hours in the control was 62 individuals and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00% of the test concentration were 66, 60, 61, 59 and 58 individuals, respectively. The population growth rate of the test organisms in the control was 0.63 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.64, 0.62, 0.63, 0.62 and 0.61, respectively.

4.3 Percentage inhibition growth

4.3.1 Discharge treated water - > 32 psu (Table 9)

The percent inhibition in average growth rate of the test organisms in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were respectively 7.34, 1.75, 1.12, 0.80 and -0.80 percent at the end of the experiment.

4.3.2 Discharge treated water - 3-32 psu (Table 12)

The percent inhibition in average growth rate of the test organisms in the 6.25, 12.50, 25.00, 50.00 and

100.00 % of the test concentration were respectively -6.06, 3.64, 1.21, 3.91 and 5.66 percent at the end of the experiment.

4.4 Plotting of the concentration response curve (Fig. 1, 2)

The raw data of population growth rate from the each test concentration of the > 32 and 3-32 psu discharge treated water was plotted on the graph.

4.5 NOEC, LOEC and ECx (Table 13)

For >32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 96h-EC50 were equal to or greater than 100.00% discharge treated water at the end of the experiment.

4.6 Other observation

Any abnormal appearance or behavior of the rotifer, *Brachionus plicatilis* during the experiment period was not observed.

5. DISCUSSION AND CONCLUSION

The population growth rate of the rotifer, *B. plicatilis* on the > 32 and 3-32 psu discharge treated water from the BioVioletTM was assessed.

For the > 32 psu discharge treated water, the population growth rate of the test organisms in the control was 0.59 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.57, 0.58, 0.58, 0.58 and 0.59, respectively (Table 8). The highest percent inhibition at the end of experiment was 6.25 % of the test concentration. However, percent inhibition of the other test concentration was very low, such as, -0.80~1.75 % (Table 9).

For the 3-32 psu discharge treated water, the population growth rate of the test organisms in the control was 0.63 and those that in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were 0.64, 0.62, 0.63, 0.62 and 0.61, respectively (Table 11). The percent inhibition in average growth rate of the test organisms in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration were respectively -6.06, 3.64, 1.21, 3.91 and 5.66 percent at the end of the experiment (Table 12).

For the >32 and 3-32 psu discharge treated water, values of NOEC, LOEC and 96h-EC50 based on population growth rate at the end of experiment were equal to or higher than 100.00 % discharge treated water (Table 13).

Therefore, it was assessed that the > 32 psu and 3-32 psu discharge treated water from the BioVioletTM did not have chronic toxic effects on the population growth of the rotifer, *B. plicatilis*.

6. REFERENCE

ASTM. 2004. Standard guide for acute toxicity test with the rotifer *Brachionus*. E-1440-91. 8pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

Janssen, C.R., G. Persoone and T.W. Snell. 1994. Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus*. Aquatic Toxicology, 28, 243-258.

TABLES

Table 1. For > 32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	6.15	6.24	6.52	6.61	6.21	6.12
Final	5.56	5.49	5.00	4.88	5.21	5.67

Table 2. For > 32 psu discharge treated water, pH value of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	7.74	7.75	7.74	7.73	7.72	7.70
Final	8.06	8.09	8.04	8.06	8.02	8.08

Table 3. For > 32 psu discharge treated water, temperature (˚C) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	25.30	24.90	24.80	24.90	24.80	24.80
Final	25.40	25.20	25.10	25.00	25.40	25.40

Table 4. For 3-32 psu discharge treated water, dissolved oxygen value (mg/L) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	5.51	5.47	5.82	5.67	5.17	5.06
Final	5.63	5.02	5.97	5.72	5.50	5.68

Table 5. For 3-32 psu discharge treated water, pH value of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	7.80	7.72	7.84	7.83	7.83	7.84
Final	7.72	7.92	8.03	8.06	8.04	8.07

Table 6. For 3-32 psu discharge treated water, temperature (˚C) of test concentration series at the beginning and the end of the test using *Brachionus plicatilis*

Concentration	Control	6.25 %	12.50 %	25.00 %	50.00 %	100.00 %
Initial	25.50	25.50	25.20	25.30	25.50	25.50
Final	25.00	24.70	25.10	25.00	25.00	24.90

Table 7. Mean number of the *Brachionus plicatilis*, exposed to > 32 psu discharge treated water for 96 hours

Concentration	**Control**					**6.25%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	17	29	52	5	5	14	27	48
STDEV(±)	0.00	0.00	2.24	1.87	3.14	0.00	0.00	1.37	3.09	1.83
Concentration	**12.50%**					**25.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	14	28	51	5	5	15	31	52
STDEV(±)	0.00	0.00	2.64	3.49	4.12	0.00	0.00	2.34	2.05	2.15
Concentration	**50.00%**					**100.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	15	28	52	5	5	16	28	53
STDEV(±)	0.00	0.00	2.45	2.50	3.59	0.00	0.00	2.58	2.90	4.19

*STDEV: Standard deviation

Table 8. Mean population growth rate for the *Brachionus plicatilis*, exposed to > 32 psu discharge treated water for 96 hours

Concentration	**Control**					**6.25%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.59	0.58	0.59	0.00	0.00	0.52	0.57	0.57
STDEV(±)	0.00	0.00	0.07	0.02	0.02	0.00	0.00	0.05	0.04	0.01
Concentration	**12.50%**					**25.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.52	0.57	0.58	0.00	0.00	0.54	0.60	0.58
STDEV(±)	0.00	0.00	0.10	0.04	0.02	0.00	0.00	0.08	0.02	0.01
Concentration	**50.00%**					**100.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.55	0.58	0.58	0.00	0.00	0.56	0.58	0.59
STDEV(±)	0.00	0.00	0.08	0.03	0.02	0.00	0.00	0.08	0.04	0.02

*STDEV: Standard deviation

Table 9. Percent inhibition in average growth rate of *Brachionus plicatilis*, at the each test concentration of treated > 32 psu ballast water during the experiment period

Elapsed time (h)	**Concentration (%)**				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	0.00	0.00	0.00	0.00	0.00
48	13.13	13.13	10.61	7.58	6.06
72	4.36	1.45	-7.27	1.16	1.16
96	7.34	1.75	1.12	0.80	-0.80

Table 10. Number of the *Brachionus plicatilis*, exposed to 3-32 psu discharge treated water for 96 hours

Concentration	**Control**					**6.25%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	22	38	62	5	5	23	34	66
STDEV(±)	0.00	0.00	6.55	6.38	4.86	0.00	0.00	3.78	3.26	3.73
Concentration	**12.50%**					**25.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	19	31	60	5	5	24	35	61
STDEV(±)	0.00	0.00	3.41	5.58	4.58	0.00	0.00	1.64	4.17	4.52
Concentration	**50.00%**					**100.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Mean	5	5	23	37	59	5	5	23	33	58
STDEV(±)	0.00	0.00	5.18	3.37	5.23	0.00	0.00	4.95	6.36	3.50

*STDEV: Standard deviation

Table 11. Population growth rate for the *Brachionus plicatilis*, exposed to 3-32 psu discharge treated water for 96 hours

Concentration	**Control**					**6.25%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.71	0.67	0.63	0.00	0.00	0.75	0.64	0.64
STDEV(±)	0.00	0.00	0.18	0.06	0.02	0.00	0.00	0.08	0.03	0.01
Concentration	**12.50%**					**25.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.67	0.60	0.62	0.00	0.00	0.79	0.64	0.63
STDEV(±)	0.00	0.00	0.09	0.06	0.02	0.00	0.00	0.03	0.04	0.02
Concentration	**50.00%**					**100.00%**				
Elapsed time (hour)	**0**	**24**	**48**	**72**	**96**	**0**	**24**	**48**	**72**	**96**
Growth rate	0.00	0.00	0.75	0.67	0.62	0.00	0.00	0.76	0.62	0.61
STDEV(±)	0.00	0.00	0.12	0.03	0.02	0.00	0.00	0.11	0.07	0.01

*STDEV: Standard deviation

Table 12. Percent inhibition in average growth rate of *Brachionus plicatilis*, at the each test concentration of treated 3-32 psu ballast water during the experiment period

Elapsed time (h)	**Concentration (%)**				
	6.25	**12.50**	**25.00**	**50.00**	**100.00**
24	0.00	0.00	0.00	0.00	0.00
48	-5.36	11.49	-11.11	-5.36	-6.51
72	10.24	19.61	9.59	3.49	14.60
96	-6.06	3.64	1.21	3.91	5.66

Table 13. NOEC, LOEC and 96h-EC50 values calculated from population growth data of animal at the end of the experiment

Test substance	**NOEC (%)**	**LOEC (%)**	**96h-EC50 (%)**	**95 % confidence of limit (%)**	
				lower	**upper**
> 32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-
3-32 psu discharge treated water	100.0	> 100.0	> 100.0	-	-

* -: no data; > 100.0: greater than 100.0 %

FIGURES

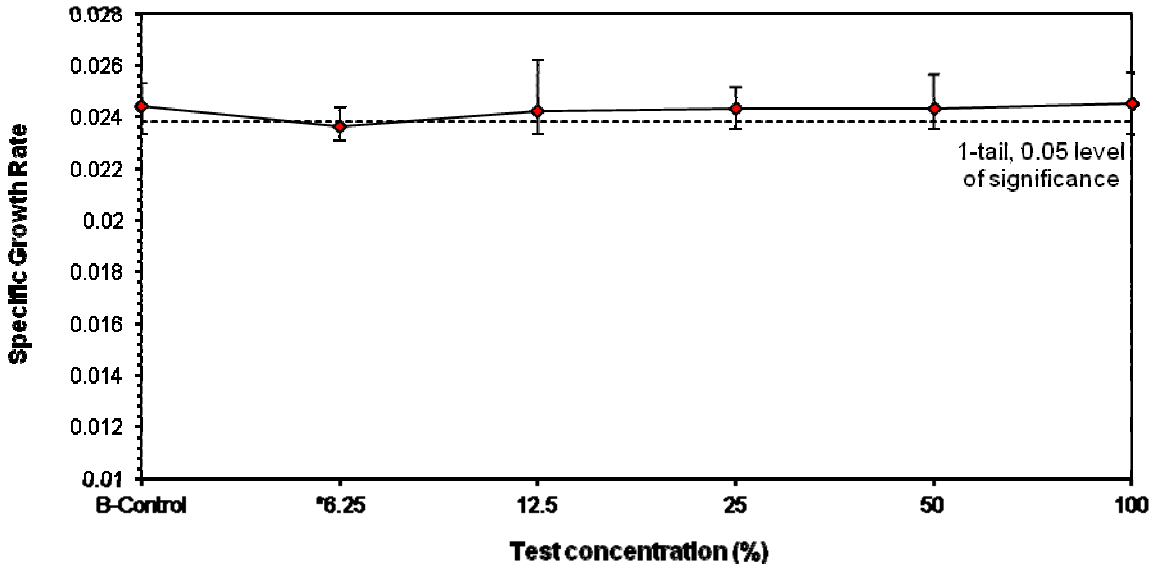

Fig. 1. Plot of the concentration-response (population growth) data from the each test concentration for > 32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

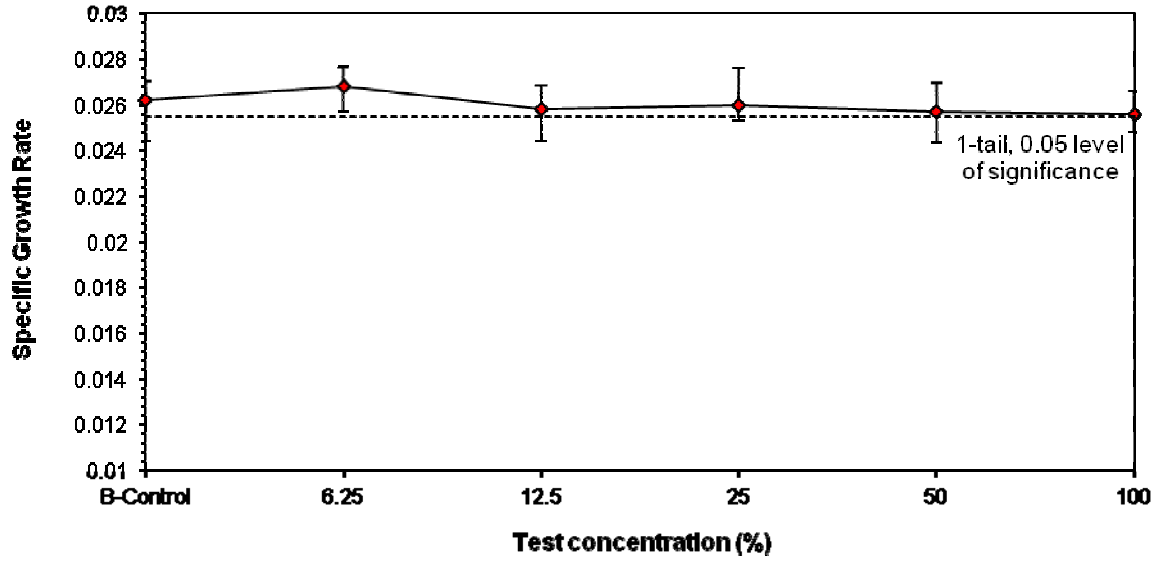

Fig. 2. Plot of the concentration-response (population growth) data from the each test concentration for 3-32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

ANNEX 1

Preparation of test substance and procedures of the experiment

Fig. 1. For aquatic toxicity test, collection of the control (untreated) and treated de-ballasting water from the BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

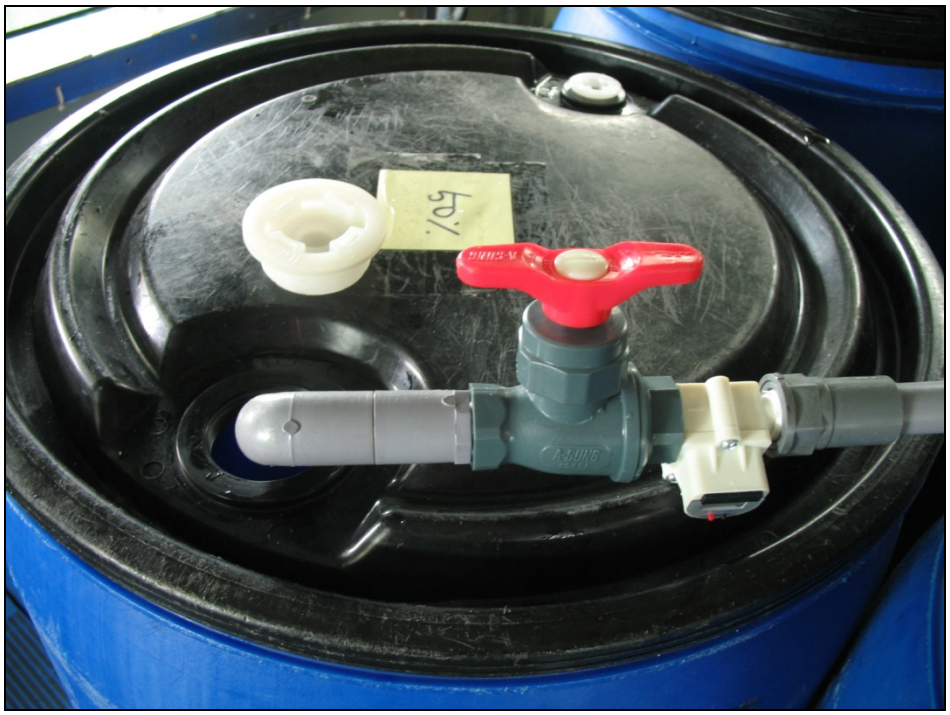

Fig. 3. Preparation of test concentration diluted with non-treated seawater on treated seawater through flow-meter.

Fig. 4. For the survival test using the rotifer, *Brachionus plicatilis*, final preparation of test substance was filtered by 0.45 μm membrane filter.

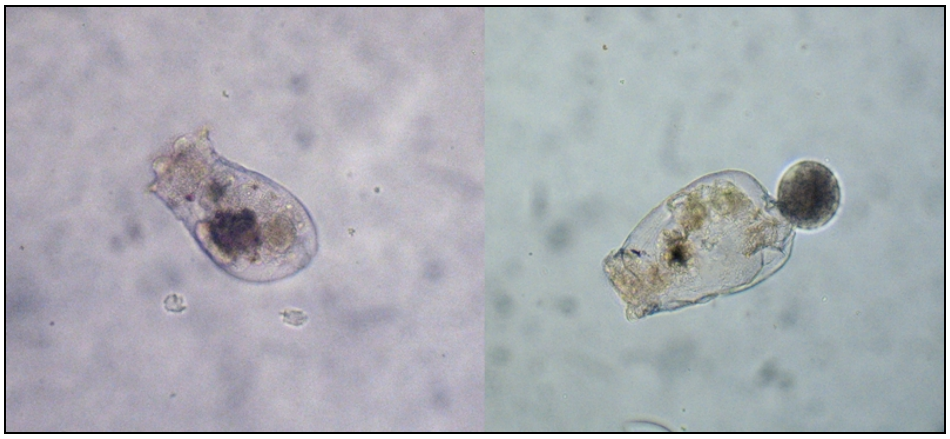

Fig. 5. Test organisms in this study: Neonates (left) and adult of female (right) of rotifer, *Brachionus plicatilis*.

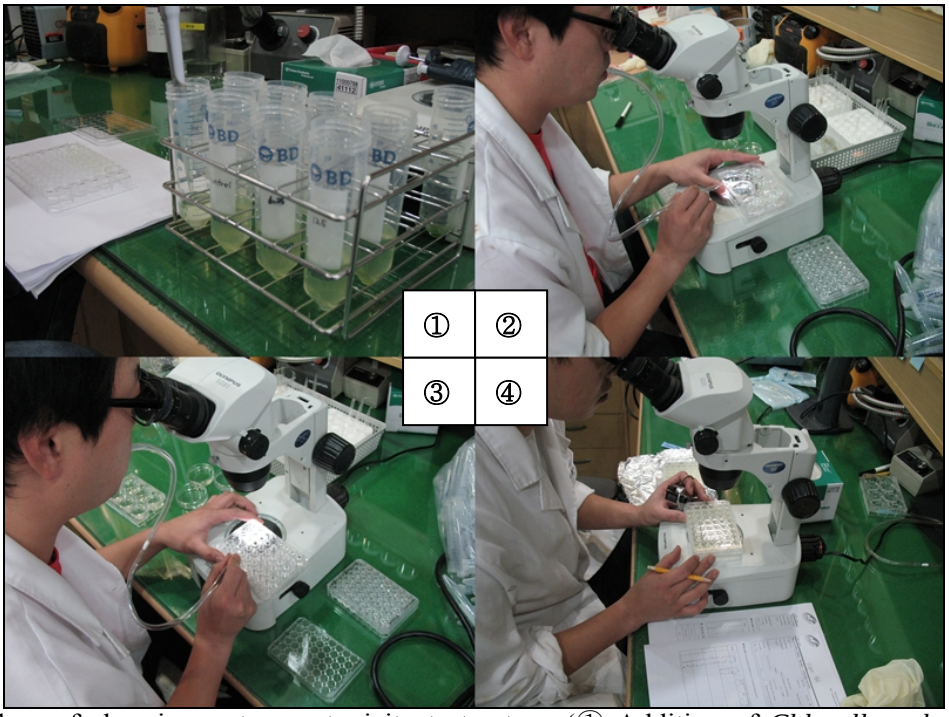

Fig. 6. Procedure of chronic crustacean toxicity test set up (① Addition of *Chlorella vulgaris* (1×10^6 cells/mL) on the test substance; ② Separation of test organisms from hatching chamber; ③ Placing of test organisms on the test vessel by modified Pasteur pipette; ④ Observation and counting of test organisms).

2.3.2 ANNEX 2

The olive flounder fish, *Paralichthys olivaceus*, Chronic Toxicity Test

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FINAL REPORT

Chronic Aquatic Toxicity For > 32 & 3-32 psu Discharge treated water of BioViolet™

- The olive flounder fish, *Paralichthys olivaceus*, Chronic Toxicity Test

Study Name: BioViolet™

Study No: BW- DBWT1107-KS

Marine Eco-Technology Institute Co., Ltd.

485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

GUARANTEE OF ASSURANCE

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Quality Assurance personnel Sang-Hee Shin (signature)
Marine Eco-technology Institute Co., Ltd.

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Type of Inspections	Date of inspection	Date Reported to Test Facility Management and Study Director
Study Plan	2011 .02.28	2011 .02.28
Test organisms	2011 .10.11 / 11.01	2011 .10.11 / 11.01
Storage of Test substance	2011 .10.10 / 10.31	2011 .10.11 / 11.01
Preparation of Test substance	2011 .10.11 / 11.01	2011 .10.11 / 11.01
Exposure	2011 .10.11 / 11.01	2011 .10.11 / 11.01
Observation	2011 .10.12 / 11.02	2011 .10.12 / 11.02
Record of raw data	2011 .10.12 / 11.02	2011 .10.12 / 11.02
Draft of final report	2011 .12.23	2011 .12.23
Final report	2011 .12.30	2011 .12.30

It is assurance that the procedure in this final report was appropriately carried out and the description of the results was reflected raw data from the study.

2011. 12. 30.

Quality Assurance personnel Sang-Hee Shin (signature)
Marine Eco-technology Institute Co., Ltd.

CONTENTS

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1. SUMMARY

The purpose of this study was to assess the chronic toxic effects of the > 32 and 3-32 psu discharge treated water by the BioVioletTM on the embryo and sac-fry stages of olive flounder, *Paralichthys olivaceus.*

The test substance was divided into two different salinities, the one was > 32 psu discharge treated water and the other was 3-32 psu discharge treated water. Non-discharge treated water was used as control and dilution water. 30 fertilized eggs of olive flounder fish, *Paralichthys olivaceus* were exposed at the various test concentrations (0.00 % (non-discharge treated water), 6.25, 12.50, 25.00, 50.00 and 100.00 % (discharge treated water)) for 7 days. The mortality of the test organisms was daily observed and recorded. The raw data of living test organisms from this experiment was used for calculation of NOEC, LOEC, 7d-LC50 values.

For the > 32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and those were 27, 28, 28, 27 and 27 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean number of the living larvae was 22 individuals in the control and those were 20, 21, 21, 19 and 13 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean survival proportion of olive flounder, *P. olivaceus* at the end of the experiment was 74.4 percent in the control and those were 65.6, 70.0, 71.1, 64.4 and 44.4 percent in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Values of NOEC, LOEC, 7d-LC25 and 7d-LC50 at the end of experiment were 50.00, 100.00 %, 80.50% and higher than 100.00 %.

For the 3-32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and those were 29, 28, 27, 28 and 27 individuals of in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean number of the living larvae was 23 individuals in the control and those were 20, 22, 20, 26 and 26 individuals of in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean survival proportion of olive flounder, *P. olivaceus* at the end of the experiment was 77.8 percent in the control and those were 66.7, 74.4, 67.8, 85.6 and 87.8 percent in 6.25, 12.50, 25.00, 50.00, 100.00 % of the test concentration, respectively. Values of NOEC, LOEC and 7d-LC50 at the end of experiment were equal to or greater than 100.00 %.

Therefore, it was assessed that the > 32 psu discharge treated water by the BioVioletTM had chronic toxic effects on the survival of the olive flounder, *Paralichthys olivaceus*.

2. TIMETABLE FOR STUDY

2.1 For > 32 psu discharge treated water

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 11. 01
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01
Evaluation of mortality	2011. 11. 01 ~ 11. 08
Experimental completion	2011. 11. 08
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

2.2 For 3-32 psu discharge treated water

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 10. 11
Collect of sample	2011. 10. 10
Experimental start	2011. 10. 11
Exposure date	2011. 10. 11
Evaluation of mortality	2011. 10. 11 ~ 10. 18
Experimental completion	2011. 10. 18
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

3. STATISTICAL PROCEDURES

For > 32 and 3-32 psu discharge treated water, normal distribution of the data was tested by Shapiro-Wilk's Test ($p > 0.05$) and equal variance of the data was tested by Bartlett's Test. Hypothesis test was conducted by Dunnett's Test and differences were considered to be significant at $\alpha=0.05$. And then LC25 and 7d-LC50 values of the end point were estimated by Maximum Likelihood-Probit.

TOXCALC 5.0 program (Tidepool scientific software, USA) was used for above statistic analysis.

4. RESULTS

4.1 Dissolved oxygen, pH, Temperature and Salinity

4.1.1 Discharge treated water - > 32 psu (Table 1~4)

For the > 32 psu discharge treated water by the BioViolet^{TM}, DO value was ranged from 4.86 to 5.43 mg/L at the beginning of the experiment and from 5.22 to 6.06 mg/L at the end of the experiment. pH value was ranged 7.70 to 7.81 at the beginning of the experiment and was ranged from 7.75 to 7.79 at the end of the experiment. Mean temperature was 19.95 ± 0.20 ˚C (Mean ± S.D.) and mean salinity was 33.68 ± 0.37 psu (Mean ± S.D.).

4.1.2 Discharge treated water - 3-32 psu (Table 5~8)

For the 3-32 psu discharge treated water by the BioViolet^{TM}, DO value was ranged from 6.26 to 6.77 mg/L at the beginning of the experiment and from 5.55 to 6.45 mg/L at the end of the experiment. pH value was ranged from 7.83 to 7.86 at beginning of the experiment and from 7.46 to 7.76 at the end of the experiment. Mean temperature was 19.56 ± 0.22 ˚C (Mean ± S.D.) and Mean salinity was 20.45 ± 1.62 psu (Mean ± S.D.).

4.2 Mortality / Survival

4.2.1 Embryo stages (Table 9, 10)

For the > 32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and 27, 28, 28, 27 and 27 individuals of a 30 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Numbers hatched was equal with numbers of the living eggs. The all living eggs hatched for 3 days after the beginning of the experiment.

For the 3-32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and 29, 28, 27, 28 and 27 individuals of a 30 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Numbers hatched was equal with numbers of the living eggs. The all living eggs hatched for 3 days after the beginning of the experiment.

4.2.2 Larval stages (Table 11, 12)

For the > 32 psu discharge treated water, mean number of the living larvae was 22 individuals of a 30 individuals in the control and 20, 21, 21, 19 and 13 individuals in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. For the 3-32 psu discharge treated water, mean number of the living larvae was 23 individuals of a 30 individuals and 20, 22, 20, 26 and 26 individuals of a 30 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively.

4.2.3 Overall survival proportions (Table 13, 14)

For the > 32 psu discharge treated water, mean survival proportions of olive flounder, *Paralichthys olivaceus* at the end of the experiment was 74.4 percent in the control and those were 65.6, 70.0, 71.1, 64.4 and 44.4 percent in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively.

For the 3-32 psu discharge treated water, mean survival proportions of olive flounder, *Paralichthys olivaceus* at the end of the experiment was 77.8 percent in the control and those were 66.7, 74.4, 67.8, 85.6 and 87.8 percent in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentrations, respectively.

4.3 Length and weight

Total length and dry weight are shown in the Annex 2. For the > 32 psu discharge treated water, mean total length and dry weight of the larval *Paralichthys olivaceus* was 2.59 ± 0.02 mm (Mean ± S.D. mm) and 0.1 mg. For the 3-32 psu discharge treated water, mean total length and wet weight of the larval *Paralichthys olivaceus* was 2.57 ± 0.02 mm (Mean ± S.D. mm)) and 0.1 mg.

4.4 Plotting of the concentration response curve (Fig. 1, 2)

The survival data on the each test concentration for the > 32 and 3-32 psu discharge treated water was plotted on the graph.

4.5 NOEC, LOCE, LCx (Table 15)

For >32 psu discharge treated water, values of NOEC, LOEC, 7d-LC25 and 7d-LC50 at the end of experiment were 50.00, 100.00 %, 80.50% and higher than 100.00 %.

For 3-32 psu discharge treated water, values of NOEC, LOEC, 7d-LC25 and 7d-LC50 at the end of experiment were equal to or higher than 100.00 %.

4.6 Other observation

Abnormality of body form and/or pigmentation was not observed. Abnormal behavior and abnormalities, such as hyperventilation, uncoordinated swimming, and atypical quiescence was not observed. Embryonic development of all survival test organisms was normal.

5. DISCUSSION AND CONCLUSION

The mortality of embryo and sac-fry stages of olive founder, *Paralichthys olivaceus* on the > 32 and 3-32 psu discharge treated water by the BioViolet^{TM} was tested for 7 days.

For the > 32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and those were 27, 28, 28, 27 and 27 individuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean number of the living larvae was 22 individuals in the control and those were 20, 21, 21, 19 and 13ndividuals in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean survival proportions of olive flounder, *Paralichthys olivaceus* at the end of the experiment was 74.4 percent in the control and those were 65.6, 70.0, 71.1, 64.4 and 44.4 percent in the 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Values of NOEC, LOEC and 7d-LC50 at the end of experiment were 50.00, 100.00 and higher than 100.00 % discharge treated water.

For the 3-32 psu discharge treated water, mean number of the living eggs on the embryo stages was 28 individuals of a 30 individuals in the control and those were 29, 28, 27, 28 and 27 individuals of in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean number of the living larvae was 23 individuals in the control and those were 20, 22, 20, 26 and 26 individuals of in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Mean survival proportion of olive flounder, *Paralichthys olivaceus* at the end of the experiment was 77.8 percent in the control and those were 66.7, 74.4, 67.8, 85.6 and 87.8 percent in 6.25, 12.50, 25.00, 50.00 and 100.00 % of the test concentration, respectively. Values of NOEC, LOEC and 7d-LC50 at the end of experiment were equal to or higher than 100.00 % discharge treated water.

Therefore, it was assessed that the > 32 psu discharge treated water by the BioViolet^{TM} had chronic toxic effects on the survival of the olive flounder, *Paralichthys olivaceus*.

6. REFERENCE

OECD guidelines for the testing of chemicals-212. Fish, Short-term Toxicity Test on the Embryo and Sac-fry Stages. 20pp.

U.S. STA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.STA-821-R-02-013.

TABLES

Table 1. For >32 psu discharge treated water, dissolved oxygen values (mg/L) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	5.32	-	4.96	-	5.43	-	5.13	-	4.86	-	4.86	-
1	-	5.93	-	5.31	-	5.71	-	5.61	-	5.10	-	5.23
2	6.72	6.43	5.72	6.11	6.13	6.37	5.73	6.34	5.83	6.12	4.87	5.89
3	-	6.12	-	6.63	-	6.21	-	5.69	-	5.48	-	6.02
4	5.73	5.06	5.47	6.54	5.87	6.37	5.84	6.27	5.91	6.18	5.82	6.13
5	-	6.11	-	6.21	-	6.24	-	6.21	-	6.04	-	6.44
6	5.82	6.66	4.83	6.06	5.39	6.37	5.64	6.29	5.54	5.98	5.93	6.56
7	-	6.06	-	5.65	-	5.51	-	5.36	-	5.22	-	5.51

Table 2. For >32 psu discharge treated water, pH values of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	7.70	-	7.73	-	7.81	-	7.72	-	7.77	-	7.73	-
1	-	7.79	-	7.75	-	7.84	-	7.78	-	7.82	-	7.76
2	7.61	7.64	7.54	7.59	7.61	7.64	7.58	7.62	7.61	7.67	7.60	7.64
3	-	7.68	-	7.70	-	7.73	-	7.64	-	7.76	-	7.71
4	7.68	7.63	7.73	7.80	7.69	7.75	7.68	7.77	7.69	7.75	7.78	7.80
5	-	7.69	-	7.87	-	7.81	-	7.81	-	7.79	-	7.84
6	7.72	7.82	7.72	7.80	7.73	7.81	7.71	7.80	7.74	7.80	7.75	7.86
7	-	7.79	-	7.75	-	7.78	-	7.76	-	7.76	-	7.77

Table 3. For >32 psu discharge treated water, temperature (˚C) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	19.90	-	19.80	-	19.80	-	19.80	-	19.80	-	19.90	-
1	-	19.80	-	19.80	-	19.80	-	19.90	-	19.90	-	19.90
2	20.50	20.00	20.50	19.80	20.60	19.80	20.50	19.80	20.30	19.80	20.40	19.80
3	-	19.80	-	19.80	-	19.90	-	19.90	-	19.90	-	19.90
4	19.90	19.80	19.90	19.90	19.80	20.00	19.90	20.00	19.80	19.90	19.90	19.90
5	-	19.80	-	19.90	-	20.10	-	19.90	-	20.00	-	19.90
6	19.90	20.20	19.80	20.10	19.90	20.20	19.90	20.20	19.80	20.20	19.80	20.10
7	-	19.70	-	19.80	-	19.90	-	19.90	-	19.90	-	19.90

Table 4. For >32 psu discharge treated water, salinity (psu) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	33.90	-	33.90	-	33.90	-	33.60	-	33.80	-	33.80	-
1	-	34.00	-	33.90	-	33.90	-	33.60	-	33.80	-	33.80
2	33.00	33.80	33.50	33.60	33.80	33.80	33.80	33.80	33.80	33.80	33.80	33.80
3	-	32.80	-	32.90	-	33.90	-	33.90	-	33.10	-	33.20
4	32.80	32.60	32.90	33.80	33.90	33.80	33.90	33.80	33.10	33.90	33.20	33.80
5	-	33.80	-	32.90	-	33.70	-	33.80	-	33.90	-	33.80
6	33.80	33.80	33.00	33.90	33.70	34.00	33.80	33.60	33.90	33.90	33.80	34.00
7	-	33.90	-	34.00	-	34.10	-	34.00	-	34.20	-	34.20

Table 5. For 3-32 psu treated ballast, dissolved oxygen values (mg/L) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	6.77	-	6.51	-	6.43	-	6.36	-	6.26	-	6.67	-
1	-	6.19	-	6.21	-	6.03	-	6.12	-	6.07	-	6.11
2	4.81	6.05	5.89	6.22	5.27	5.69	5.12	5.72	5.78	5.68	6.01	5.79
3	-	6.31	-	6.42	-	6.88	-	6.12	-	5.95	-	6.32
4	4.17	5.53	4.72	6.03	4.69	5.34	4.10	5.11	5.43	6.12	4.28	5.24
5	-	6.01	-	6.12	-	5.08	-	5.66	-	5.96	-	5.18
6	5.80	6.62	6.03	6.82	5.77	6.10	5.42	6.26	6.12	6.33	5.88	6.60
7	-	5.92	-	6.45	-	5.56	-	5.55	-	5.98	-	5.71

Table 6. For 3-32 psu discharge treated water, pH values of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	7.83	-	7.84	-	7.86	-	7.86	-	7.86	-	7.84	-
1	-	7.74	-	7.78	-	7.73	-	7.76	-	7.73	-	7.65
2	7.34	7.65	7.40	7.71	7.41	7.71	7.46	7.70	7.45	7.67	7.38	7.64
3	-	7.46	-	7.56	-	7.65	-	7.50	-	7.56	-	7.51
4	7.32	7.63	7.43	7.74	7.34	7.53	7.29	7.54	7.53	7.61	7.42	7.66
5	-	7.66	-	7.67	-	7.61	-	7.61	-	7.59	-	7.68
6	7.42	7.64	7.62	7.75	7.55	7.68	7.44	7.72	7.51	7.64	7.60	7.71
7	-	7.46	-	7.70	-	7.57	-	7.66	-	7.58	-	7.76

Table 7. For 3-32 psu discharge treated water, temperature (˚C) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	19.80	-	19.10	-	19.30	-	19.40	-	19.40	-	19.80	-
1	-	19.30	-	19.40	-	19.60	-	19.70	-	19.70	-	19.60
2	19.60	20.10	19.60	19.80	19.70	20.00	19.70	20.10	19.80	20.00	19.50	19.90
3	-	19.00	-	19.40	-	19.60	-	19.60	-	19.70	-	19.30
4	19.80	19.70	19.50	19.40	19.60	19.50	19.60	19.70	19.60	19.70	19.60	19.70
5	-	19.60	-	19.60	-	19.40	-	19.60	-	19.50	-	19.60
6	19.20	19.60	19.30	19.40	19.60	19.60	19.60	19.60	19.50	19.60	19.40	19.50
7	-	19.60	-	19.50	-	19.50	-	19.50	-	19.50	-	19.50

Table 8. For 3-32 psu discharge treated water, salinity (psu) of test concentration series during the experiment period

Elapsed time (day)	Control		6.25%		12.50%		25.00%		50.00%		100.00%	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
0	20.10	-	20.20	-	20.20	-	20.20	-	20.20	-	20.00	-
1	-	20.50	-	20.40	-	20.30	-	20.20	-	33.30	-	20.20
2	20.10	20.70	20.30	20.40	20.20	20.30	19.90	20.20	20.10	20.20	20.00	20.20
3	-	20.90	-	19.80	-	19.90	-	20.30	-	20.20	-	20.20
4	20.10	20.40	20.30	20.00	20.30	20.40	20.30	20.40	20.20	20.20	20.10	20.30
5	-	20.30	-	20.10	-	20.40	-	20.30	-	20.30	-	20.40
6	20.00	20.30	20.20	20.20	20.10	20.10	20.20	20.10	20.20	20.30	20.10	20.00
7	-	20.50	-	20.70	-	20.70	-	20.70	-	20.20	-	20.60

Table 9. Number of living eggs on embryo stages of *Paralichthys olivaceus* exposed to > 32 psu discharge treated water

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	30	30	30	30	30	30	30	30	30	30	30	30
1	30	30	30	30	30	29	30	30	30	30	30	30
2	30	28	29	29	29	28	28	28	29	29	29	29
3	29	27	29	28	29	26	27	27	29	29	26	28
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**	**1**	**2**	**3**	**Mean**
0	30	30	30	30	30	30	30	30	30	30	30	30
1	29	30	30	30	30	29	29	29	30	30	29	30
2	28	28	30	29	30	29	29	29	27	27	26	27
3	27	28	30	28	27	24	29	27	27	27	26	27

Table 10. Number of living eggs on the embryo stages of *Paralichthys olivaceus* exposed to 3-32 psu discharge treated water

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	30	30	30	30	30	30	30	30	30	30	30	30
1	30	27	29	29	30	30	29	30	30	30	30	30
2	30	27	29	29	30	30	29	30	30	30	30	30
3	30	25	28	28	29	30	29	29	30	30	25	28
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	30	30	30	30	30	30	30	30	30	30	30	30
1	30	30	29	30	30	30	27	29	28	28	28	28
2	30	29	29	29	29	30	27	29	28	28	27	28
3	30	24	28	27	29	30	26	28	28	27	27	27

Table 11. Number of survival larvae of *Paralichthys olivaceus* exposed to > 32 psu discharge treated water

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
4	27	23	23	24	23	20	19	21	25	26	20	24
5	26	22	23	24	21	20	18	20	24	26	19	23
6	24	22	23	23	21	20	18	20	24	21	19	21
7	22	22	23	22	21	20	18	20	24	20	19	21
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
4	19	22	24	22	17	22	19	19	14	16	13	14
5	19	21	24	21	17	22	19	19	14	15	11	13
6	19	21	24	21	17	22	19	19	14	15	11	13
7	19	21	24	21	17	22	19	19	14	15	11	13

Table 12. Number of survival larvae of *Paralichthys olivaceus* exposed to 3-32 psu discharge treated water

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
4	28	22	22	24	26	29	28	28	26	27	24	26
5	28	22	22	24	24	22	23	23	24	22	22	23
6	28	22	22	24	20	21	20	20	24	22	22	23
7	26	22	22	23	19	21	20	20	23	22	22	22
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
4	22	21	23	22	28	29	22	26	28	27	27	27
5	22	20	22	21	28	28	21	26	27	26	27	27
6	22	20	20	21	28	28	21	26	26	26	27	26
7	22	20	19	20	28	28	21	26	26	26	27	26

Table 13. Overall survival proportions of olive flounder, *Paralichthys olivaceus* exposed to test concentration series of the > 32 psu discharge treated water during the experiment period

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	100.0	100.0	100.0	100.0	96.7	100.0	98.9	100.0	100.0	100.0	100.0
2	100.0	93.3	96.7	96.7	90.0	83.3	76.7	83.3	96.7	93.3	80.0	90.0
3	93.3	76.7	80.0	83.3	86.7	66.7	63.3	72.2	86.7	93.3	66.7	82.2
4	90.0	76.7	76.7	81.1	76.7	66.7	63.3	68.9	83.3	86.7	66.7	78.9
5	86.7	73.3	76.7	78.9	70.0	66.7	60.0	65.6	80.0	86.7	63.3	76.7
6	80.0	73.3	76.7	76.7	70.0	66.7	60.0	65.6	80.0	70.0	63.3	71.1
7	73.3	73.3	76.7	74.4	70.0	66.7	60.0	65.6	80.0	66.7	63.3	70.0
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	96.7	100.0	100.0	98.9	100.0	96.7	96.7	97.8	100.0	100.0	96.7	98.9
2	90.0	86.7	96.7	91.1	90.0	73.3	63.3	75.6	66.7	63.3	66.7	65.6
3	63.3	80.0	80.0	74.4	60.0	73.3	63.3	65.6	53.3	60.0	46.7	53.3
4	63.3	73.3	80.0	72.2	56.7	73.3	63.3	64.4	46.7	53.3	43.3	47.8
5	63.3	70.0	80.0	71.1	56.7	73.3	63.3	64.4	46.7	50.0	36.7	44.4
6	63.3	70.0	80.0	71.1	56.7	73.3	63.3	64.4	46.7	50.0	36.7	44.4
7	63.3	70.0	80.0	71.1	56.7	73.3	63.3	64.4	46.7	50.0	36.7	44.4

Table 14. Overall survival proportions of olive flounder, *Paralichthys olivaceus* exposed to test concentration series of the 3-32 psu discharge treated water during the experiment period

Elapsed time (day)	Control				6.25%				12.50%			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	90.0	96.7	95.6	100.0	100.0	96.7	98.9	100.0	100.0	100.0	100.0
2	100.0	90.0	96.7	95.6	100.0	100.0	96.7	98.9	100.0	100.0	100.0	100.0
3	100.0	86.7	93.3	93.3	93.3	100.0	96.7	96.7	100.0	100.0	83.3	94.4
4	93.3	73.3	73.3	80.0	86.7	96.7	93.3	92.2	86.7	90.0	80.0	85.6
5	93.3	73.3	73.3	80.0	80.0	73.3	76.7	76.7	80.0	73.3	73.3	75.6
6	93.3	73.3	73.3	80.0	66.7	70.0	66.7	67.8	80.0	73.3	73.3	75.6
7	86.7	73.3	73.3	77.8	63.3	70.0	66.7	66.7	76.7	73.3	73.3	74.4
Elapsed time (day)	**25.00%**				**50.00%**				**100.00%**			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
1	100.0	100.0	96.7	98.9	100.0	100.0	90.0	96.7	93.3	93.3	93.3	93.3
2	100.0	96.7	96.7	97.8	96.7	100.0	90.0	95.6	93.3	93.3	90.0	92.2
3	100.0	80.0	93.3	91.1	96.7	100.0	86.7	94.4	93.3	90.0	90.0	91.1
4	73.3	70.0	76.7	73.3	93.3	96.7	73.3	87.8	93.3	90.0	90.0	91.1
5	73.3	66.7	73.3	71.1	93.3	93.3	70.0	85.6	90.0	86.7	90.0	88.9
6	73.3	66.7	66.7	68.9	93.3	93.3	70.0	85.6	86.7	86.7	90.0	87.8
7	73.3	66.7	63.3	67.8	93.3	93.3	70.0	85.6	86.7	86.7	90.0	87.8

Table 15. NOEC, LOEC, 7d-LC25 and 7d-LC50 value calculated from survival data at the end of the experiment

Test substance	NOEC (%)	LOEC (%)	7d-LC25 (%)	95 % confidence of limit (%)		7d-LC50 (%)	95 % confidence of limit (%)	
				lower	upper		lower	upper
> 32 psu discharge treated water	50.00	100.0	80.50	60.65	92.72	> 100.0	-	-
3-32 psu discharge treated water	100.0	>100.0	-	-	-	> 100.0	-	-

* -: no data; > 100: greater than 100 %

FIGURES

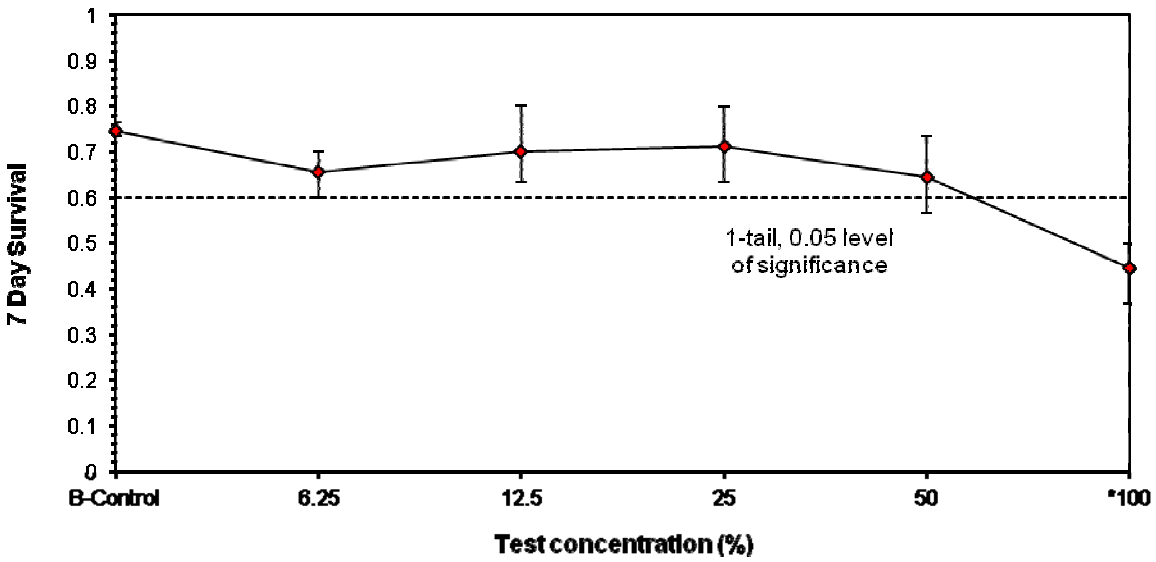

Fig. 1. Plot of the concentration-response data from the each test concentration of > 32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

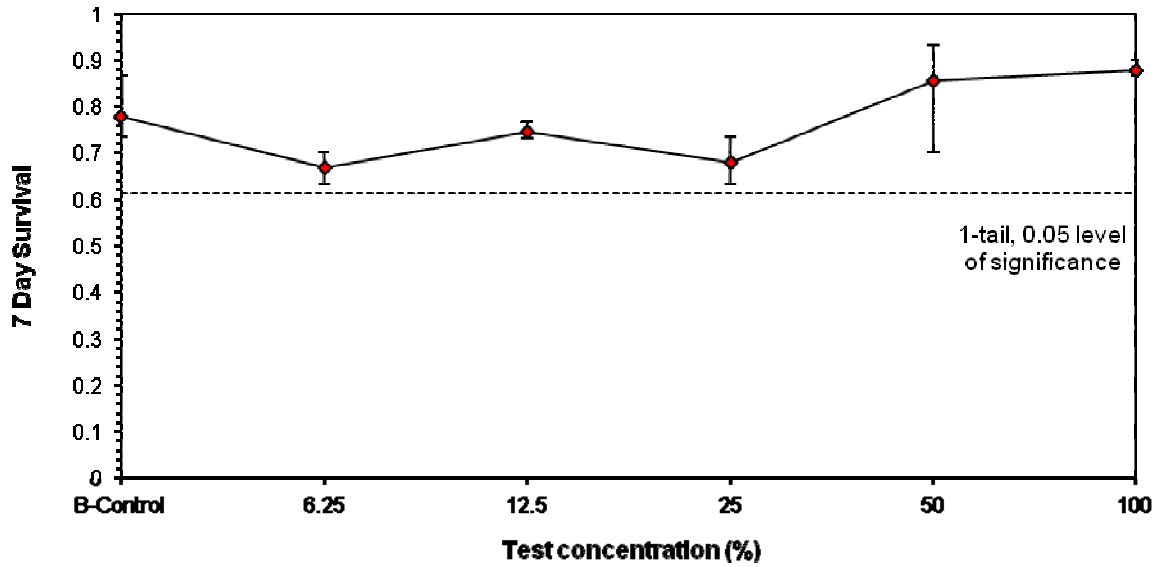

Fig. 2. Plot of the concentration-response data from the each test concentration of 3-32 psu discharge treated water. The dotted line was represented as 0.05 level of significant and vertical bar was represented maximum and minimum.

ANNEX 1

Preparation of test substance and procedure of the experiment

Fig. 1. For aquatic toxicity test, collection of the control (untreated) and treated de-ballasting water from BioVioletTM.

Fig. 2. Transfer collected samples to the constant room (4 ± 1 ˚C).

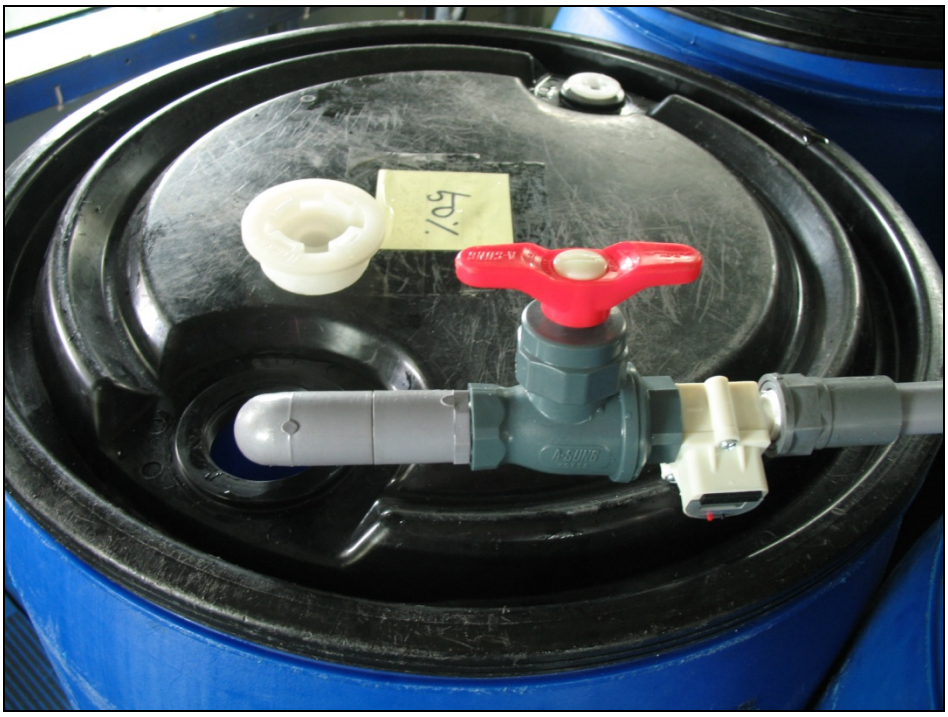

Fig. 3. Preparation of test concentration diluted with non-treated seawater on treated seawater through flow-meter.

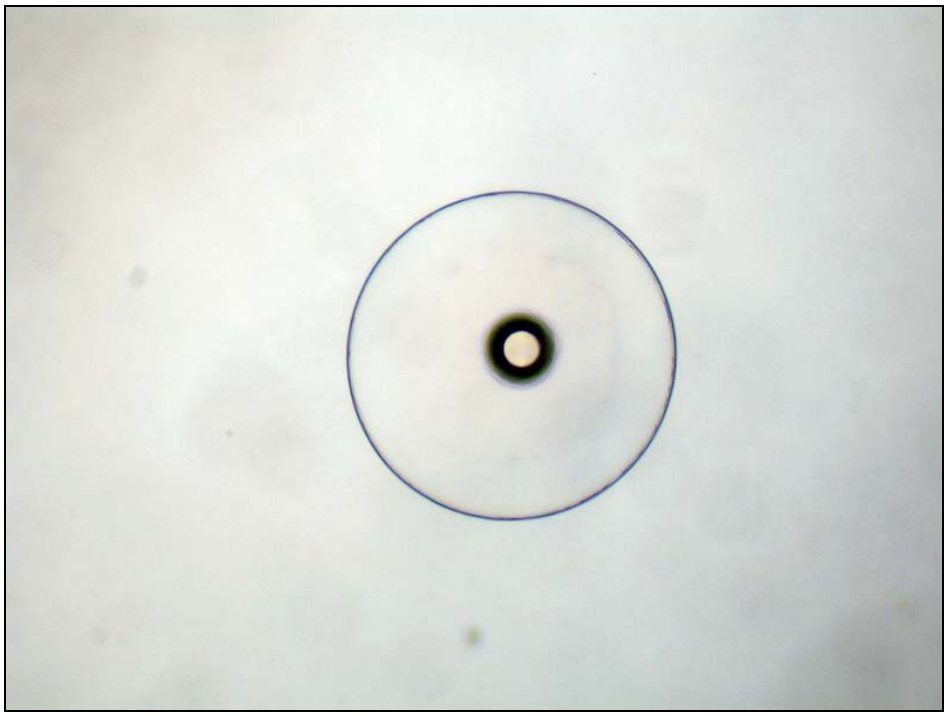

Fig. 4. Test organisms in this study: Fertilized eggs of olive flounder, *Paralichthys olivaceus*.

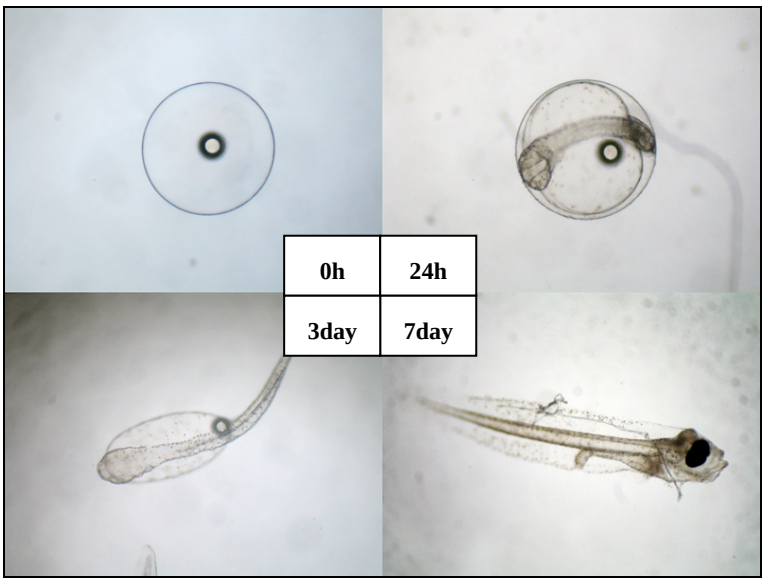

Fig. 5. Test organisms in this study: Development fertilized eggs to larvae of olive flounder, *Paralichthys olivaceus*.

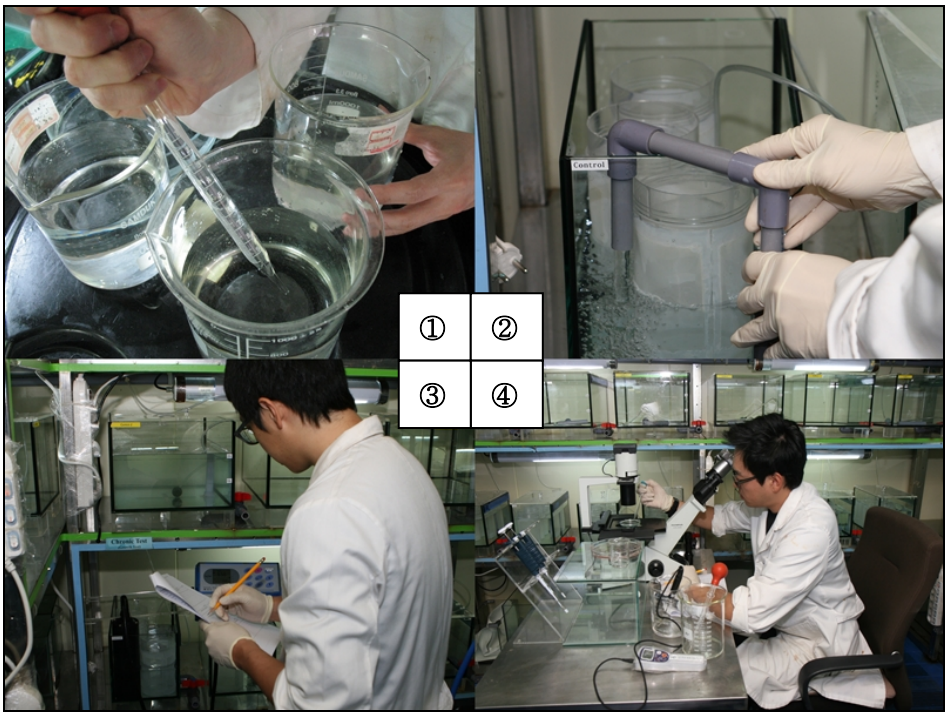

Fig. 6. Procedure of chronic fish toxicity test set up (① Sorting the fertilized eggs; ② Test vessels set up; ③ Check the water quality of the test substance and recording ④ Measurement of length of the organisms)

ANNEX 2

▶ Total length and dry weight of larval olive flounder, *Paralichthys olivaceus*, at the end of the experiment for > 32 psu discharge treated water by the BioViolet™

▷ **Concentration :** Control						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.7	0.1
2	2.5	0.1	2.5	0.1	2.5	0.1
3	2.5	0.1	2.5	0.1	2.5	0.1
4	2.5	0.1	2.6	0.1	2.6	0.1
5	2.5	0.1	2.6	0.1	2.6	0.1
6	2.5	0.1	2.6	0.1	2.6	0.1
7	2.5	0.1	2.6	0.1	2.6	0.1
8	2.6	0.1	2.5	0.1	2.8	0.1
9	2.6	0.1	2.5	0.1	2.6	0.1
10	2.6	0.1	2.6	0.1	2.5	0.1
11	2.5	0.1	2.5	0.1	2.9	0.1
12	2.5	0.1	2.8	0.1	2.7	0.1
13	2.5	0.1	2.6	0.1	2.7	0.1
14	2.7	0.1	2.6	0.1	2.7	0.1
15	2.5	0.1	2.7	0.1	2.6	0.1
16	2.5	0.1	2.7	0.1	2.5	0.1
17	2.6	0.1	2.5	0.1	2.5	0.1
18	2.8	0.1	2.8	0.1	2.6	0.1
19	2.5	0.1	2.5	0.1	2.6	0.1
20	2.8	0.1	2.5	0.1	2.5	0.1
21	2.5	0.1	2.7	0.1	2.7	0.1
22	2.6	0.1	2.6	0.1	2.6	0.1
23					2.8	0.1
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 6.25 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.7	0.1	2.7	0.1
2	2.5	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.6	0.1	2.5	0.1
4	2.6	0.1	2.5	0.1	2.5	0.1
5	2.5	0.1	2.6	0.1	2.6	0.1
6	2.5	0.1	2.6	0.1	2.6	0.1
7	2.7	0.1	2.5	0.1	2.7	0.1
8	2.7	0.1	2.6	0.1	2.6	0.1
9	2.7	0.1	2.6	0.1	2.6	0.1
10	2.8	0.1	2.6	0.1	2.7	0.1
11	2.7	0.1	2.7	0.1	2.7	0.1
12	2.5	0.1	2.6	0.1	2.8	0.1
13	2.6	0.1	2.5	0.1	2.5	0.1
14	2.6	0.1	2.5	0.1	2.8	0.1
15	2.6	0.1	2.8	0.1	2.6	0.1
16	2.5	0.1	2.5	0.1	2.5	0.1
17	2.6	0.1	2.9	0.1	2.5	0.1
18	2.8	0.1	2.5	0.1	2.7	0.1
19	2.5	0.1	2.8	0.1		
20	2.9	0.1	2.5	0.1		
21	2.8	0.1				
22						
23						
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 12.50 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.6	0.1	2.6	0.1	2.5	0.1
2	2.5	0.1	2.6	0.1	2.6	0.1
3	2.5	0.1	2.6	0.1	2.6	0.1
4	2.5	0.1	2.6	0.1	2.6	0.1
5	2.5	0.1	2.5	0.1	2.5	0.1
6	2.6	0.1	2.5	0.1	2.5	0.1
7	2.5	0.1	2.5	0.1	2.7	0.1
8	2.5	0.1	2.6	0.1	2.5	0.1
9	2.6	0.1	2.7	0.1	2.7	0.1
10	2.5	0.1	2.6	0.1	2.6	0.1
11	2.5	0.1	2.8	0.1	2.7	0.1
12	2.7	0.1	2.5	0.1	2.8	0.1
13	2.7	0.1	2.8	0.1	2.6	0.1
14	2.5	0.1	2.7	0.1	2.8	0.1
15	2.5	0.1	2.5	0.1	2.5	0.1
16	2.6	0.1	2.6	0.1	2.5	0.1
17	2.6	0.1	2.6	0.1	2.6	0.1
18	2.6	0.1	2.5	0.1	2.6	0.1
19	2.7	0.1	2.5	0.1	2.6	0.1
20	2.5	0.1	2.7	0.1		
21	2.6	0.1				
22	2.6	0.1				
23	2.5	0.1				
24	2.5	0.1				
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 25.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.6	0.1	2.5	0.1
2	2.6	0.1	2.6	0.1	2.6	0.1
3	2.5	0.1	2.6	0.1	2.6	0.1
4	2.5	0.1	2.6	0.1	2.5	0.1
5	2.6	0.1	2.6	0.1	2.5	0.1
6	2.5	0.1	2.6	0.1	2.5	0.1
7	2.5	0.1	2.5	0.1	2.5	0.1
8	2.5	0.1	2.5	0.1	2.5	0.1
9	2.6	0.1	2.8	0.1	2.7	0.1
10	2.6	0.1	2.5	0.1	2.5	0.1
11	2.7	0.1	2.7	0.1	2.5	0.1
12	2.5	0.1	2.7	0.1	2.5	0.1
13	2.6	0.1	2.7	0.1	2.8	0.1
14	2.6	0.1	2.5	0.1	2.5	0.1
15	2.7	0.1	2.6	0.1	2.5	0.1
16	2.7	0.1	2.6	0.1	2.6	0.1
17	2.6	0.1	2.5	0.1	2.5	0.1
18	2.5	0.1	2.5	0.1	2.6	0.1
19	2.5	0.1	2.5	0.1	2.6	0.1
20			2.6	0.1	2.6	0.1
21			2.7	0.1	2.7	0.1
22					2.5	0.1
23					2.6	0.1
24					2.5	0.1
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 50.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.7	0.1
2	2.5	0.1	2.5	0.1	2.6	0.1
3	2.6	0.1	2.6	0.1	2.5	0.1
4	2.5	0.1	2.7	0.1	2.5	0.1
5	2.5	0.1	2.8	0.1	2.6	0.1
6	2.7	0.1	2.5	0.1	2.6	0.1
7	2.7	0.1	2.6	0.1	2.6	0.1
8	2.7	0.1	2.6	0.1	2.8	0.1
9	2.6	0.1	2.6	0.1	2.6	0.1
10	2.5	0.1	2.5	0.1	2.5	0.1
11	2.6	0.1	2.8	0.1	2.7	0.1
12	2.5	0.1	2.5	0.1	2.5	0.1
13	2.6	0.1	2.7	0.1	2.6	0.1
14	2.8	0.1	2.5	0.1	2.8	0.1
15	2.5	0.1	2.6	0.1	2.5	0.1
16	2.6	0.1	2.6	0.1	2.7	0.1
17	2.7	0.1	2.6	0.1	2.5	0.1
18			2.5	0.1	2.6	0.1
19			2.8	0.1	2.6	0.1
20			2.5	0.1		
21			2.6	0.1		
22			2.8	0.1		
23						
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 100.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.5	0.1
2	2.5	0.1	2.6	0.1	2.6	0.1
3	2.6	0.1	2.6	0.1	2.5	0.1
4	2.6	0.1	2.7	0.1	2.5	0.1
5	2.5	0.1	2.7	0.1	2.6	0.1
6	2.5	0.1	2.5	0.1	2.6	0.1
7	2.7	0.1	2.7	0.1	2.5	0.1
8	2.5	0.1	2.6	0.1	2.6	0.1
9	2.7	0.1	2.5	0.1	2.7	0.1
10	2.7	0.1	2.5	0.1	2.5	0.1
11	2.5	0.1	2.5	0.1	2.6	0.1
12	2.6	0.1	2.5	0.1		
13	2.8	0.1	2.5	0.1		
14	2.6	0.1	2.6	0.1		
15			2.6	0.1		
16						
17						
18						
19						
20						
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▶ Total length and dry weight of larval olive flounder, *Paralichthys olivaceus*, at the end of the experiment for 3-32 psu discharge treated water by the BioViolet™

▷ **Concentration :** Control						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.9	0.1	2.6	0.1
2	2.5	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.5	0.1	2.6	0.1
4	2.7	0.1	2.5	0.1	2.6	0.1
5	2.5	0.1	2.5	0.1	2.5	0.1
6	2.5	0.1	2.6	0.1	2.5	0.1
7	2.5	0.1	2.6	0.1	2.6	0.1
8	2.5	0.1	2.6	0.1	2.7	0.1
9	2.6	0.1	2.6	0.1	2.6	0.1
10	2.5	0.1	2.5	0.1	2.7	0.1
11	2.5	0.1	2.7	0.1	2.8	0.1
12	2.5	0.1	2.5	0.1	2.6	0.1
13	2.7	0.1	2.7	0.1	2.5	0.1
14	2.5	0.1	2.6	0.1	2.6	0.1
15	2.5	0.1	2.5	0.1	2.8	0.1
16	2.6	0.1	2.8	0.1	2.8	0.1
17	2.6	0.1	2.6	0.1	2.7	0.1
18	2.5	0.1	2.6	0.1	2.6	0.1
19	2.7	0.1	2.7	0.1	2.5	0.1
20	2.6	0.1	2.7	0.1	2.5	0.1
21	2.8	0.1	2.7	0.1	2.5	0.1
22	2.9	0.1	2.6	0.1	2.6	0.1
23	2.6	0.1				
24	2.6	0.1				
25	2.6	0.1				
26	2.6	0.1				
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 6.25 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.7	0.1	2.6	0.1
2	2.8	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.5	0.1	2.6	0.1
4	2.6	0.1	2.5	0.1	2.5	0.1
5	2.8	0.1	2.5	0.1	2.6	0.1
6	2.5	0.1	2.5	0.1	2.6	0.1
7	2.5	0.1	2.5	0.1	2.6	0.1
8	2.5	0.1	2.5	0.1	2.7	0.1
9	2.6	0.1	2.5	0.1	2.6	0.1
10	2.5	0.1	2.5	0.1	2.5	0.1
11	2.6	0.1	2.6	0.1	2.5	0.1
12	2.5	0.1	2.6	0.1	2.5	0.1
13	2.5	0.1	2.6	0.1	2.8	0.1
14	2.7	0.1	2.6	0.1	2.8	0.1
15	2.5	0.1	2.5	0.1	2.6	0.1
16	2.5	0.1	2.7	0.1	2.7	0.1
17	2.5	0.1	2.6	0.1	2.5	0.1
18	2.5	0.1	2.7	0.1	2.6	0.1
19	2.6	0.1	2.6	0.1	2.5	0.1
20			2.7	0.1	2.5	0.1
21			2.5	0.1		
22						
23						
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 12.50 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.6	0.1
2	2.5	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.5	0.1	2.6	0.1
4	2.5	0.1	2.5	0.1	2.5	0.1
5	2.6	0.1	2.5	0.1	2.5	0.1
6	2.6	0.1	2.5	0.1	2.7	0.1
7	2.6	0.1	2.5	0.1	2.7	0.1
8	2.5	0.1	2.5	0.1	2.7	0.1
9	2.5	0.1	2.6	0.1	2.8	0.1
10	2.5	0.1	2.5	0.1	2.6	0.1
11	2.5	0.1	2.5	0.1	2.5	0.1
12	2.6	0.1	2.5	0.1	2.5	0.1
13	2.7	0.1	2.5	0.1	2.5	0.1
14	2.5	0.1	2.7	0.1	2.5	0.1
15	2.7	0.1	2.5	0.1	2.6	0.1
16	2.5	0.1	2.5	0.1	2.5	0.1
17	2.5	0.1	2.6	0.1	2.5	0.1
18	2.6	0.1	2.8	0.1	2.6	0.1
19	2.5	0.1	2.5	0.1	2.6	0.1
20	2.5	0.1	2.7	0.1	2.5	0.1
21	2.7	0.1	2.5	0.1	2.9	0.1
22	2.6	0.1	2.6	0.1	2.7	0.1
23	2.5	0.1				
24						
25						
26						
27						
28						
29						
30						
Mean	2.6	0.1	2.5	0.1	2.6	0.1

▷ **Concentration :** 25.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.6	0.1
2	2.6	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.7	0.1	2.6	0.1
4	2.6	0.1	2.5	0.1	2.5	0.1
5	2.5	0.1	2.5	0.1	2.6	0.1
6	2.5	0.1	2.5	0.1	2.5	0.1
7	2.7	0.1	2.5	0.1	2.7	0.1
8	2.5	0.1	2.5	0.1	2.5	0.1
9	2.6	0.1	2.5	0.1	2.7	0.1
10	2.5	0.1	2.5	0.1	2.6	0.1
11	2.5	0.1	2.7	0.1	2.5	0.1
12	2.5	0.1	2.5	0.1	2.5	0.1
13	2.5	0.1	2.5	0.1	2.6	0.1
14	2.5	0.1	2.6	0.1	2.6	0.1
15	2.5	0.1	2.6	0.1	2.8	0.1
16	2.6	0.1	2.5	0.1	2.6	0.1
17	2.5	0.1	2.5	0.1	2.5	0.1
18	2.5	0.1	2.5	0.1	2.5	0.1
19	2.5	0.1	2.6	0.1	2.5	0.1
20	2.7	0.1	2.8	0.1		
21	2.5	0.1				
22	2.5	0.1				
23						
24						
25						
26						
27						
28						
29						
30						
Mean	2.5	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 50.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.5	0.1	2.5	0.1	2.6	0.1
2	2.5	0.1	2.5	0.1	2.6	0.1
3	2.5	0.1	2.6	0.1	2.6	0.1
4	2.5	0.1	2.5	0.1	2.6	0.1
5	2.7	0.1	2.6	0.1	2.6	0.1
6	2.6	0.1	2.5	0.1	2.6	0.1
7	2.5	0.1	2.5	0.1	2.5	0.1
8	2.5	0.1	2.5	0.1	2.5	0.1
9	2.5	0.1	2.5	0.1	2.5	0.1
10	2.5	0.1	2.5	0.1	2.6	0.1
11	2.5	0.1	2.5	0.1	2.7	0.1
12	2.5	0.1	2.5	0.1	2.5	0.1
13	2.5	0.1	2.5	0.1	2.5	0.1
14	2.5	0.1	2.5	0.1	2.5	0.1
15	2.6	0.1	2.5	0.1	2.8	0.1
16	2.5	0.1	2.6	0.1	2.7	0.1
17	2.5	0.1	2.6	0.1	2.5	0.1
18	2.5	0.1	2.8	0.1	2.5	0.1
19	2.5	0.1	2.5	0.1	2.7	0.1
20	2.5	0.1	2.5	0.1	2.7	0.1
21	2.5	0.1	2.5	0.1	2.6	0.1
22	2.5	0.1	2.6	0.1		
23	2.5	0.1	2.7	0.1		
24	2.6	0.1	2.7	0.1		
25	2.5	0.1	2.8	0.1		
26	2.5	0.1	2.5	0.1		
27	2.5	0.1	2.6	0.1		
28	2.5	0.1	2.5	0.1		
29						
30						
Mean	2.5	0.1	2.6	0.1	2.6	0.1

▷ **Concentration :** 100.00 %						
Replicate	**1**		**2**		**3**	
No.	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**	**Length (mm)**	**Dry weight (mg)**
1	2.6	0.1	2.5	0.1	2.7	0.1
2	2.5	0.1	2.5	0.1	2.5	0.1
3	2.5	0.1	2.5	0.1	2.6	0.1
4	2.5	0.1	2.5	0.1	2.6	0.1
5	2.5	0.1	2.6	0.1	2.5	0.1
6	2.7	0.1	2.6	0.1	2.5	0.1
7	2.5	0.1	2.7	0.1	2.5	0.1
8	2.5	0.1	2.6	0.1	2.5	0.1
9	2.5	0.1	2.5	0.1	2.6	0.1
10	2.5	0.1	2.7	0.1	2.5	0.1
11	2.6	0.1	2.5	0.1	2.6	0.1
12	2.7	0.1	2.7	0.1	2.6	0.1
13	2.5	0.1	2.8	0.1	2.5	0.1
14	2.5	0.1	2.5	0.1	2.6	0.1
15	2.5	0.1	2.6	0.1	2.7	0.1
16	2.6	0.1	2.6	0.1	2.8	0.1
17	2.5	0.1	2.9	0.1	2.7	0.1
18	2.5	0.1	2.9	0.1	2.6	0.1
19	2.5	0.1	2.6	0.1	2.5	0.1
20	2.6	0.1	2.6	0.1	2.9	0.1
21	2.6	0.1	2.8	0.1	2.6	0.1
22	2.8	0.1	2.6	0.1	2.8	0.1
23	2.7	0.1	2.5	0.1	2.5	0.1
24	2.7	0.1	2.5	0.1	2.5	0.1
25	2.5	0.1	2.6	0.1	2.5	0.1
26	2.6	0.1	2.5	0.1	2.7	0.1
27					2.6	0.1
28						
29						
30						
Mean	2.6	0.1	2.6	0.1	2.6	0.1

3. REFERENCE

INTERNATIONAL STANDARD

ISO 10253

Second edition
2006-04-15

Water quality — Marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*

Qualité de l'eau — Essai d'inhibition de la croissance des algues marines avec Skeletonema costatum *et* Phaeodactylum tricornutum

Reference number
ISO 10253:2006(E)

OECD GUIDELINE FOR TESTING OF CHEMICALS

Adopted by the Council on 17th July 1992

Fish, Acute Toxicity Test

INTRODUCTION

1. This new version of the guideline, originally adopted in 1981 and first updated in 1984, is based on a proposal from the United Kingdom to reduce the numbers of fish in tests of acute aquatic toxicity. The proposal was discussed at a meeting of OECD experts convened at Medmenham (United Kingdom) in November 1988.

2. The main differences in comparison with the earlier versions are the reduction in group-size allowing the use of seven fish per group, the extension of the concentration range by allowing a spacing factor of 2.2 instead of 2 and the introduction of a limit test at 100 mg/l of test substance.

PRINCIPLE OF THE TEST

3. The fish are exposed to the test substance preferably for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentrations which kill 50 per cent of the fish (LC50) are determined where possible.

INFORMATION ON THE TEST SUBSTANCE

4. It is necessary to know the water solubility of the substance under the conditions of the test. A reliable analytical method for the quantification of the substance in the test solutions must also be available.

5. Useful information includes the structural formula, purity of the substance, stability in water and light, pK_a, P_{ow}, vapour pressure and results of a test for ready biodegradability (see Guideline 301). Solubility and vapour pressure can be used to calculate Henry's constant which will indicate if losses of the test substance may occur.

VALIDITY OF THE TEST

6. For a test to be valid the following conditions should be fulfilled:

- the mortality in the control(s) should not exceed 10 per cent (or one fish if less than ten are used) at the end of the test;

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Adopted:
21st September 1998

OECD GUIDELINE FOR TESTING OF CHEMICALS

Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages

INTRODUCTION

1. This Short-term Toxicity Test on Fish Embryo and Sac-Fry Stages is a short-term test in which the life stages from the newly fertilized egg to the end of the sac-fry stage are exposed. No feeding is provided in the embryo and sac-fry test, and the test should thus be terminated while the sac-fry are still nourished from the yolk-sac. It is based on a proposal from Denmark which was discussed at an OECD meeting of experts convened at Medmenham (United Kingdom) in December 1991.

2. This guideline is intended to define lethal, and to a limited extent, sublethal effects of chemicals on the specific stages and species tested.

3. This Guideline does not replace Guideline 210 but it would provide useful information in that it could (a) form a bridge between lethal and sublethal tests, (b) be used as a screening test for either a Full Early Life Stage test (Guideline 210) or for chronic toxicity and (c) be used for testing species where husbandry techniques are not sufficiently advanced to cover the period of change from endogenous to exogenous feeding.

4. It should be borne in mind that only tests incorporating all stages of the life-cycle of fish are generally liable to give an accurate estimate of the chronic toxicity of chemicals to fish, and that any reduced exposure with respect to life stages may reduce the sensitivity and thus underestimate the chronic toxicity. It is therefore expected that the embryo and sac-fry test would be less sensitive than the Full Early Life Stage test (Guideline 210), particularly with respect to chemicals with high lipophilicity ($\log P_{ow} > 4$) and chemicals with a specific mode of toxic action. However smaller differences in sensitivity between the two tests would be expected for chemicals with a non-specific, narcotic mode of action (1).

5. Prior to the publication of this Guideline, most experience with this embryo and sac-fry test has been with the freshwater fish *Danio rerio* Hamilton-Buchanan (Teleostei, Cyprinidae - common name zebrafish). More detailed guidance on test performance for this species is therefore given in Annex 1. This does not preclude the use of other species for which experience is also available (Table 1).

Designation: E 1440 – 91 (Reapproved 2004)

Standard Guide for Acute Toxicity Test with the Rotifer Brachionus[1]

This standard is issued under the fixed designation E 1440; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide describes procedures for obtaining laboratory data concerning the acute toxicity of chemicals and aqueous effluents released into fresh, estuarine, or marine waters. Acute toxicity is measured by exposing *Brachionus* newly hatched from cysts to a series of toxicant concentrations under controlled conditions. This guide describes a test for using *B. calyciflorus*, a fresh water rotifer, and the Appendix describes modifications of this test for estuarine and marine waters using *B. plicatilis*. These procedures lead to an estimation of acute toxicity, including the concentration expected to kill 50 % of the test rotifers (LC50) in 24 h. Procedures not specifically stated in this guide should be conducted in accordance with Guide E 729 and Guide E 1192.

1.2 Modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, the results of tests conducted using modified procedures might not be comparable to rotifer acute tests that follow the protocol described here. Comparison of the results using modified procedures might provide useful information concerning new concepts and procedures for conducting acute toxicity tests on chemicals and aqueous effluents.

1.3 This guide is organized as follows:

1.4 These procedures are applicable to most chemicals, either individually or in formulations, commercial products, or mixtures. This guide can also be used to conduct investigations of the effects on rotifer survival of pH, hardness, and salinity and on materials such as aqueous effluents, leachates, oils, particulate matter, sediments, and surface waters. This guide might not be appropriate for materials with high oxygen demand, with high volatility, subject to rapid biological or chemical transformation, or that readily sorb to test chambers.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* For specific hazards statements, see Section 8.

2. Referenced Documents

2.1 *ASTM Standards:* [2]

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)

E 729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians[3]

E 943 Terminology Relating to Biological Effects and Environmental Fate[3]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians[3]

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *rotifer cyst*—a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology.

Current edition approved April 1, 2004. Published April 2004. Originally approved in 1991. Last previous edition approved in 1998 as E 1440 – 91 (1998).

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Withdrawn.

ELSEVIER

Aquatic Toxicology 28 (1994) 243–258

AQUATIC TOXICOLOGY

Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus*

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Abstract

The development and potential use of a 4-day static renewal test [4-day Life Table (LT) test] and a 3-day static test [3-day Population Growth (PG) test] with the freshwater rotifers *Brachionus calyciflorus* are described. For both bioassays, test animals are obtained by hatching cysts which eliminates the need for the culturing and maintenance of the organisms. The toxicity of copper (Cu), pentachlorophenol (PCP), 3,4-dichloroaniline (DCA) and lindane was assessed using the developed methods. The NOEC's, based on the test endpoint r_m, obtained with the 4-day LT test were 0.0025, 0.4, 5 and 20 mg/l for Cu, PCP, DCA and lindane, respectively. Similar results were obtained with the 3-day PG tests for which NOEC's of 0.005, 0.8, 20 and 10 mg/l, respectively, were recorded. The mean cv between replicated 3-day PG tests was 10%, indicating a good intra-laboratory reproducibility of the test results. For Cu and PCP, the sensitivity of *B. calyciflorus* compared favourably to chronic toxicity tests with *Daphnia magna*, while for the other two compounds *B. calyciflorus* proved to be rather insensitive. Considering the increasing need for relatively short toxicity tests, the two described short- chronic bioassays could be valuable new tools for routine toxicity evaluations. The major advantages associated with these tests are: they are less labour-intensive than existing chronic tests, they can be completed within one work week, and do not require stock culturing of test organisms.

Key words: Rotifer; *Brachionus calyciflorus*; Chronic; Life table; Population growth

1. Introduction

Invertebrate short-term chronic toxicity tests have been widely applied in the evaluation of toxic hazards. The two main freshwater chronic toxicity tests with inverte-

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EPA
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Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms

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